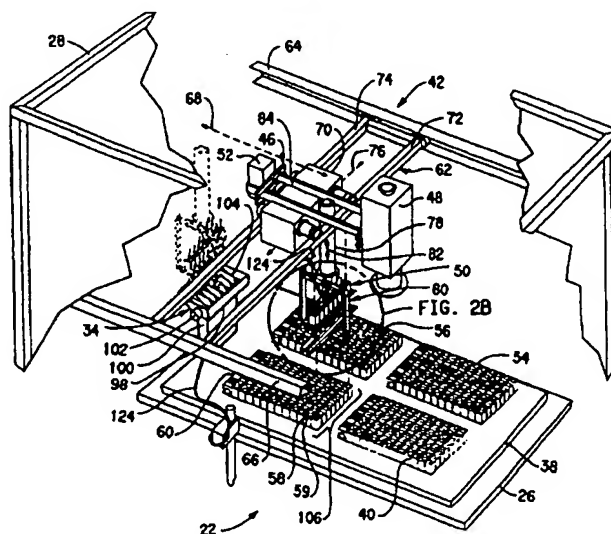




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(54) Title: <b>AGRICULTURAL PRODUCT MICROSCREEN METHOD AND APPARATUS</b>		



## (57) Abstract

A method and apparatus for screening compounds for agricultural activity has now been developed which employs e.g., intact plants grown in microtiter plates on very small amounts of plant growth media containing a test compound. In comparison to the standard greenhouse screen, the microscreen requires vastly less space, labor, and test compound. However, unlike *in vitro* screens, responses of intact plants are assayed. Using the microscreen, high-throughput screening of test compounds can be accomplished using whole plant responses as the assay.

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TITLE  
AGRICULTURAL PRODUCT MICROSCREEN  
METHOD AND APPARATUS  
BACKGROUND OF THE INVENTION

5       Herbicide candidates are normally identified either by screening test compounds for biological activity on intact plants grown in soil ("greenhouse" assays) or by screening test compounds for inhibition of specific target enzymes or other proteins *in vitro* ("in vitro" assays). In greenhouse assays, test compounds are either sprayed onto soil containing ungerminated seeds (pre-emergent application) or onto the plants themselves  
10 (post-emergent application). Greenhouse assays have a high success rate in predicting the herbicidal activity of a compound when applied in the field. Additionally, a greenhouse assay simultaneously tests for compounds which affect essentially all potential modes of herbicidal action. In other words, greenhouse assays possess a high information content. However, greenhouse testing is time- and space-consuming,  
15 requiring 2 weeks and 2,ft<sup>2</sup> of greenhouse space for each compound tested. Large amounts of labor are also required to prepare, care for, spray and score these tests. Moreover, 10 to 60 mg of a test compound is required to assess its potential herbicidal activity at normal use rates. The requirement for this amount of test compound places significant limitations on the synthesis or acquisition of compounds to be tested.

20       *In vitro* assays, by contrast, typically assay the affect of a test compound on only a specific target enzyme or protein. These targets are usually extracted proteins, and their responses to the test compounds are assayed *in vitro*. Alternatively, these targets are expressed in surrogate microorganisms and their responses are assayed by their effects on the growth or metabolism of the microorganisms. In either case, these assays have  
25 great advantages over greenhouse assays, including much lower space, labor, time and compound requirements. However, the value of these assays for predicting herbicidal activity under natural conditions is limited by complex and poorly understood processes unique to intact plants which are absent *in vitro* or in surrogate organisms. Processes such as uptake, translocation, and metabolism of test compounds cannot at present be  
30 accurately predicted and must be determined empirically by evaluating responses on intact plants. Moreover, *in vitro* screens are only capable of screening test compounds against a single or very small number of targets, thus requiring separate assays for each new target.

35       A method for screening compounds for herbicidal activity which combines the high information content and good predictive qualities of greenhouse screening with the reduced size, cost and compound requirements of *in vitro* screens, wherein high-throughput screening of test compounds can be accomplished using whole plant responses as the assay, is desirable.

Dornbos and Spencer have examined the germination and early seedling growth of three species of weeds (*Medicago sativa*, *Lolium multiflorum*, and *Abutilon theophrasti*) in 24-well tissue culture plates (Dornbos, D. L. Jr. and Spencer, G. F. *J. Chem. Ecol.* (1990), 16, 339-351). The wells in these plates were cylinders, about 1.6 cm in diameter and 1.7 cm in height, with a total volume of about 3.4 cm<sup>3</sup>. Each well contained 1 mL of 0.5% agar in water. Various amounts of test compounds were added to the surface of the agar in 1 mL of solvent (99% hexane, 1% chloroform) and the solvent was then allowed to evaporate. Seeds were placed on the agar and after 3 days, seed germination and seedling length were measured. This assay is terminated at 3 days after inoculation of the seed onto the agar, a point at which the seedlings are still largely dependent on seed reserves for growth. Thus, it is likely that the short term growth will not allow the detection of injury of certain classes of herbicides which affect photosynthetic or other processes which are not required for early seedling growth.

#### SUMMARY OF THE INVENTION

This invention pertains to a method and apparatus for evaluating at least one compound for its biological activity on plants, the method comprising (a) placing a plant growth medium, at least one compound to be evaluated, and at least one seed into contact with each other and into a container having a cross-sectional area measured in the horizontal direction of not greater than 1 cm<sup>2</sup>, the at least one seed being a seed which will germinate into a plant of the genus selected from the group consisting of *Ageratum*, *Arabidopsis*, *Arabis*, *Artemisia*, *Asrostis*, *Browallia*, *Capsella*, *Coleus*, *Cortaderia*, *Cynodon*, *Cyperus*, *Digitalis*, *Digitaria*, *Eragrostis*, *Inula*, *Ipomopsis*, *Laevis*, *Lemna*, *Nicotiana*, *Oxalis*, *Panicum*, *Petunia*, *Platycodon*, *Recta*, *Sagina*, *Santolina*, *Thymophylla*, and *Thymus*, (b) maintaining the container with the medium, at least one compound and at least one seed under conditions such that in the absence of the at least one compound the at least one seed would germinate and the resulting plant would grow, and (c) evaluating the resulting germination and plant growth. Preferred is a method wherein the at least one seed is a seed which will germinate into a plant of the genus selected from the group consisting of *Arabidopsis*, *Asrostis*, *Browallia*, *Coleus*, *Digitalis*, *Digitaria*, *Eragrostis*, *Inula*, *Nicotiana*, *Oxalis*, *Panicum* and *Petunia*. Most preferred for use in the method is a seed which will germinate into a plant selected from the group consisting of *Arabidopsis thaliana*, *Browallia speciosa*, *Coleus blumei*, *Digitaria sanguinalis*, *Nicotiana tabacum*, and *Petunia hybrida*.

The method of the present invention further comprises the mechanized placing of one and only one seed at a time into each of a plurality of containers as described above, the seed being selected from the group described above, so that each seed will be in contact with a plant growth medium and a compound to be evaluated, each container being maintained under conditions such that in the absence of the compound the seed

would germinate into a single plant and the resulting plant would grow in the container. The method further comprises the automated mechanized placing of one and only one seed at a time in each of the containers as described above. The method of the present invention further comprises providing such automated mechanical seeding by providing a supply of seeds in at least one fluidized seed bed, providing a plurality of vertical hollow tubes, each of such tubes having at its lower end an opening the largest dimensions of which is smaller than the smallest dimension of the seed, each of such tubes having means for controlling air pressure within the hollow portion of the tube to be less than, equal to or greater than the air pressure outside of the tube, the air pressure within each tube being individually controllable, positioning the plurality of tubes relative to the at least one fluidized seed beds such that the lower end of the plurality of tubes is partially immersed in the at least one fluidized seed bed, controlling the air pressure within each tube to create a vacuum within each tube sufficient to capture and hold a seed at the opening at the lower end of each tube, positioning the plurality of tubes relative to the containers such that the lower end of each tube is within a container, positioned approximately in the center of the horizontal cross section of each container, below the level of the top of the container and above the level of any contents in the container, controlling the air pressure within each tube to release the vacuum and create pressure within each tube higher than the pressure outside each tube sufficient to release and expel the seed into the container.

The method further comprises having in each container before the seed is expelled therein a substance which when contacted by the seed will inhibit or prevent further movement of the seed within the container and inhibit or prevent the seed from being expelled from the container due to air currents resulting from any excess air pressure within the tube.

The method of the present invention further comprises detecting whether each container has had placed therein one and only one seed, and correcting the seeding of any container found not to contain one and only one seed. The method further comprises such detecting being done automatically by optical scanning techniques.

The present invention further comprises apparatus for carrying out the above-described method, which apparatus is described in greater detail below.

#### BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-F. Effects of CO<sub>2</sub> concentration on growth of seven different species in microtiter plates. Plant species are indicated in each panel. Plates were maintained in plexiglass chambers at the indicated CO<sub>2</sub> concentration for 12 days except for *Digitaria* which was maintained for 7 days. After this incubation period, the chlorophyll content of the plants was determined. The mean (-■-) and coefficient of variation (- -) of the chlorophyll content per seedling are plotted. Between 32 and 48 individual seedlings

were used for each data point. These experiments demonstrate that elevated levels of CO<sub>2</sub> have a positive impact on seed germination (mean) and uniformity of plant growth (coefficient of variation) for most of the tested species.

Figure 2. Correlation of biomass and chlorophyll assays. Ninety-five *Arabidopsis* plants from a single, 14-day old microscreen plate were assayed both for biomass and chlorophyll. Each data point represents a single plant. The line was calculated by least square regression using a linear model and has a correlation coefficient,  $r^2 = 0.91$ . As can be seen, there is a high correlation between plant biomass and chlorophyll content, validating the chlorophyll assay as a positive indicator of plant growth.

Figures 3A and B. Effects of chlorsulfuron and norfluorazon on *Arabidopsis* as measured by chlorophyll and biomass assays. Plants were grown in the microscreen on the indicated level of chlorsulfuron or norfluorazon for 14 days. The biomass of individual plants was then determined. Plants were then placed back into the wells and their chlorophyll content was measured. Each point represents the mean of 8 replicates and error bars represent the standard error of the mean. As can be seen, the dose response curve that results from the biomass assay is very similar to the dose response curve resulting from the chlorophyll assay, indicating that the chlorophyll assay is an accurate measure of herbicidal activity.

Figure 4 is a schematic elevation view of the overall automated seeding system.

Figure 5A is an isometric view of the seed handling and monitoring system.

Figure 5B is a break away of a portion of the seed handling system.

Figure 6 is a section view of a seed pickup tube.

Figure 7 is an enlarged view of a row of wells in a microtiter plate as seen by a monitoring camera.

Figure 8 is an isometric view of the well illumination and correction station.

Figure 9 is a partial schematic illustration of pneumatics for the seed pickup tubes.

Figures 10A and 10B are a perspective view and section view, respectively, of a microtiter lid and egg pickup tube for use in egg placement.

#### DETAILED DESCRIPTION OF THE INVENTION

A method for screening compounds (including both synthetic chemical compounds and compounds occurring in nature) for herbicidal activity (termed the herbicide microscreen) has now been developed which combines the high information content and good predictive qualities of greenhouse screening with the reduced size, cost and compound requirements of *in vitro* screens. This screening method employs intact plants grown in small containers on very small amounts of growth media containing the test compound. In comparison to the standard greenhouse screen, the microscreen requires vastly less space, labor, and test compound. However, unlike *in vitro* screens, responses of intact plants are assayed. Using the microscreen, high-throughput screening of test

compounds can be accomplished using whole plant responses as the assay. This method for detection of compounds and molecules that display herbicidal activity is capable of screening large numbers of candidates in a format that minimizes space, materials, waste stream and labor costs.

5 In a preferred embodiment, the container is one of a plurality of containers of substantially the same dimension as each other arranged in a rectangular matrix on a single supporting plate, and the method comprises placing into each of the plurality of  
10 containers a growth medium, at least one seed and either at least one compound to be evaluated or a control material. The method exploits a physical format of testing vessels that is in common use in research laboratories and clinical settings. In the most preferred  
15 embodiment, this format, commonly referred to as a microtiter plate, generally employs a test unit comprised of a series of wells, known as microwells, wherein the footprint, or the two dimensional space that the unit occupies, is approximately 9 cm by 13 cm. This  
20 format is especially convenient in that many instruments have been developed (and are commercially available) that automate plate movement, well filling, aspirating and washing, and data collection from individual microwells. The opening of each microwell  
of the microtiter plates contemplated by this invention can be square or, preferably, circular, generally with a cross-sectional area of less than 1 cm<sup>2</sup>, preferably less than  
0.5 cm<sup>2</sup>. Although a "standard" microtiter plate is comprised of cylindrical microwells  
25 arranged in an 8 x 12 matrix that are approximately 1 cm deep and can accommodate a liquid volume of 300-400 uL, this invention also pertains to other microtiter plates wherein the microwells are deeper ("deep well" microtiter plates) and can therefore  
accommodate a larger fluid volume, or alternatively wherein there are a greater number of wells per plate (for example, Nunc 384 Well Plate; Cat. No. 242757; Nunc, Inc.,  
Naperville, IL). These other microtiter plates maintain the described 9 x 13cm footprint,  
thus facilitating automated handling of microtiter plates in support of the high-  
throughput nature of the screening method. Deeper wells accommodating a larger fluid  
volume may be employed in order to afford growth of certain plant species; greater  
30 numbers of wells per plate will increase the number of compounds that can be screened per assay plate and reduce the quantities of materials (compounds, growth media) consumed per assay.

#### Species Selection

Unless otherwise specified, the experiments described herein were conducted under the following conditions. One hundred microliters of molten 0.4% agarose in  
35 1/2x MS salt media without sugar and vitamins (Murashige, T. and Skoog, F. *Physiol. Plant.* (1962) 15, 485; hereinafter "1/2x MS") were pipetted into each well of 96 well microtiter plates, allowed to cool and harden, and one seed per well was placed on the top of the agarose using a forceps. The microtiter plates were sealed with gas permeable

tape to prevent the growth media from drying out and the plates were placed in an incubator at 25°C/21°C day/night, 16 h photoperiod (45-80  $\mu\text{mol/m}^2/\text{s}$  photosynthetically active radiation (PAR)) and 60-90% relative humidity. Plates were kept in plexiglass boxes and were visually rated 14 days after planting (DAP). CO<sub>2</sub> concentration was maintained at an artificially high level (approximately 1%) by daily addition of a 1.5 g piece of dry ice to the plexiglass boxes housing the microtiter plates.

A total of 192 species were tested for growth in 96-well microtiter plates. Species were selected for testing based primarily on the small size of their seeds. Seeds were purchased from Germania (Chicago, IL) or were propagated at Stine Research Center (DuPont Co, Newark, DE). Plates were prepared as described above and 24-32 seeds were planted per species. Germination rate and growth habits of each species were observed visually 7 and 14 DAP. All species evaluated were compared using, primarily, ratings (described below) and general growth characteristics. Those species exhibiting appropriate size, shape and uniformity were advanced for additional testing. Germination rate was a factor when species within a single genus were compared. The rating scale used was as follows:

Excellent	Ideal plant for microtiter plate growth, strong in all categories (size, shape, uniformity).
Good	Nearly ideal, but lacking in one or two categories, weaknesses can probably be overcome.
Poor	Will grow, but has glaring weaknesses which may be difficult to overcome.
Unacceptable	Will not work in microtiter plates.

Of the 192 species tested, a total of 68 species were advanced for further testing in ambient and high CO<sub>2</sub> (1%) environments. Plants held in a high CO<sub>2</sub> environment were grown in sealed plexiglass boxes and 1.5 g dry ice was added daily to achieve the 1% CO<sub>2</sub> level. From this test, 35 species (listed in Table 1) were selected which were rated good to excellent as previously described. These 35 species were tested against 18 commercial herbicides representing 10 different modes of action. Table 2 lists the herbicides and modes of action.

Table 1. Plant species selected for testing biological response to commercial herbicides.

Genus	Species	Common Name
<i>Ageratum</i>	sp.	<i>Ageratum</i> (blue danube)
<i>Arabidopsis</i>	<i>thaliana</i>	<i>Arabidopsis</i>
<i>Arabis</i>	<i>blepharophylla caucasica</i>	<i>Arabis</i> (compinkie)
<i>Artemisia</i>	<i>dracunculus</i>	Tarragon
<i>Asrostis</i>	<i>stolonifera</i>	Bentgrass



<i>Browallia</i>	<i>speciosa</i>	<i>Browallia</i> (marine bells)
<i>Browallia</i>	<i>speciosa</i>	<i>Browallia</i> (jingle bell)
<i>Capsella</i>	<i>bursa-pastoris</i>	Shepherdspurse
<i>Coleus</i>	<i>blumei</i>	<i>Coleus</i> (wizard golden)
<i>Coleus</i>	<i>blumei</i>	<i>Coleus</i> (rainbow-strain)
<i>Coleus</i>	<i>blumei</i>	<i>Coleus</i> (pink dragon)
<i>Cortaderia</i>	<i>selloana-pink</i>	Pampus Grass
<i>Cynodon</i>	<i>dactylon</i>	Bermudagrass
<i>Cyperus</i>	<i>difformis</i>	<i>Cyperus</i>
<i>Digitalis</i>	<i>purpurea</i>	<i>Digitalis</i> (white)
<i>Digitalis</i>	<i>purpurea</i>	<i>Digitalis</i> (excelsior)
<i>Digitaria</i>	<i>sanguinalis</i>	Large crabgrass
<i>Eragrostis</i>	<i>curbula</i>	Weeping lovegrass
<i>Inula</i>	<i>ensifolia</i>	Ensifolia
<i>Ipomopsis</i>	<i>elegans</i>	Capitata Gilla
<i>Laevis</i>	<i>jasione-perennis</i>	Perennis Jasione
<i>Lemna</i>	<i>minor</i>	<i>Lemna</i>
<i>Nicotiana</i>	<i>tobacum</i>	Tobacco
<i>Oxalis</i>	<i>stricta</i>	Yellow woodsorrel
<i>Panicum</i>	<i>dichotomiflorum</i>	Fall panicum
<i>Panicum</i>	<i>coloratum</i>	Kleingrass
<i>Petunia</i>	<i>hybrida</i>	<i>Petunia</i> (star joy)
<i>Petunia</i>	<i>hybrida</i>	<i>Petunia</i> (carpet mixture)
<i>Petunia</i>	<i>hybrida</i>	<i>Petunia</i> (velvet frost)
<i>Platycodon</i>	<i>grandiflorum</i>	<i>Platycodon</i>
<i>Recta</i>	<i>warrensii</i>	Potentilla
<i>Sagina</i>	<i>subulata</i>	Pilifera spergula
<i>Santolina</i>	<i>chamaecyparissus-tomentosa</i>	Chamaecyparissus
<i>Thymophylla</i>	<i>puntuloba-dyssodia</i>	Dahlborg daisy
<i>Thymus</i>	<i>vulgaris</i>	Thyme

Table 2. Commercial herbicides used for testing biological response of 35 species.

Herbicide	Mode of action
cyanazine	photosystem inhibitor
metribuzine	photosystem inhibitor
betanal	photosystem inhibitor
diquat	photosystem inhibitor
pyrizone	photosystem inhibitor
norfluorazon	carotenoid biosynthesis
fluoridone	carotenoid biosynthesis

acifluorfin	porphyrin biosynthesis inhibitor
trifluralin	mitotic inhibitor
2,4-D	auxin
quizolofop ethyl	lipid biosynthesis
dichlobenil	cellulose biosynthesis
glyphosate	amino acid biosynthesis
nicosulfuron	amino acid biosynthesis
alachlor	protein synthesis
butachlor	protein synthesis
propanil	protein synthesis
bromoxynil	respiratory inhibitor

A 10,000 ppm solution of each herbicide was prepared in dimethylsulfoxide (DMSO). Thirty microliters of each solution were dissolved in 9.97 mL 1/2x MS media to yield a 30 ppm solution. Serial dilutions of the 30 ppm solution were made to prepare 0.3 and 0.003 ppm solutions. Thirty-three microliters of the 30, 0.3 and 0.003 ppm solutions were each pipetted into wells of 96-well microtiter plates. Sixty-six microliters of 0.6% agarose in 1/2x MS media were added to each well yielding a final concentration of 10, 0.1 and 0.001 ppm in 0.4% agarose. Three plates were prepared at each concentration. Controls contained 33 uL of untreated 1/2x MS media plus 66 uL of 0.6% agarose (3 plates). One seed of each species was planted in each of 8 wells (12 species per plate). Plants were rated visually for herbicidal injury using a 0-4 rating system (see Table 4) 14 DAP. Species were rated based on one or more of the following criteria:

Biological response	Species had low sensitivity to majority of herbicides or activity overlapped directly with other species.
Germination rate	Species had <50% germination rate.
Growth habit	Species were too large or small and/or did not grow uniformly.

15

Based on results of the above test, 11 species were selected for additional testing against the 18 herbicides described in Table 2 at 30, 10 and 1 ppm using similar procedures as described above. The eleven species tested were *Arabidopsis thaliana*, *Asrostis stolonifera*, *Browallia speciosa*, *Coleus blumei*, *Digitalis purpurea*, *Digitaria sanguinalis*, *Eragrostis cymbula*, *Inula ensifolia*, *Nicotiana tobacum*, *Panicum coloratum* and *Penunia hybrida*. Plants were visually rated for injury 14 DAP as described in Table 4.

20

These eleven plant species demonstrated good sensitivity for some herbicidal modes of action at concentrations as low as 0.1 ppm, had good germination rates (> 50%) and had a suitable size, shape and uniformity for growth in a microtiter plate well. These tests also served to fine tune the concentration of herbicide necessary for detection of activity. These tests showed that 1 and 10 ppm concentrations were necessary for detecting biological activity across various plant species and herbicidal modes of action.

#### Media Selection

The eleven species selected were grown in five different media to determine which medium would be best suited for each species. The five media tested were: 1/2x MS medium (*supra*), DMG Medium (Datko et al. *Plant Physiol.* (1980) 65, 906-912) *Arabidopsis* medium without sucrose (Haughn, G. W. and Somerville, C. R. *Mol. Gen. Genet.* (1986) 204, 430-434), 1/2x Hoagland's nutrient solution (Hoagland, D. R. and Arnon, D. I., (1950) California Agricultural Experiment Station, Circular #347, 32p) and monocot medium (Armstrong, C. L. and Green, C. E. *Planta* (1985) 164, 207-214).

Table 3 shows the media that worked best for each species. Good growth of all species occurred in any of the tested media. Since 1/2x MS medium was the best medium for most species, it was selected as the universal medium for this assay, although media other than those tested can also be used.

Table 3. Growth Optimization of Eleven Species.

Species	Optimum Media
<i>Arabidopsis thaliana</i>	1/2x Hoaglands or 1/2x MS
<i>Asrostis stolonifera</i>	All media
<i>Browallia speciosa</i>	1/2x Hoaglands, DMG, <i>Arabidopsis</i>
<i>Coleus blumei</i>	All media
<i>Digitalis purpurea</i>	1/2x MS, 1/2x Hoaglands, DMG
<i>Digitaria sanguinalis</i>	All media
<i>Eragrostis cymbula</i>	All media
<i>Inula ensifolia</i>	All media
<i>Nicotiana tobacum</i>	1/2x Hoaglands or 1/2x MS
<i>Panicum coloratum</i>	1/2x MS
<i>Petunia hybrida</i>	All media

#### Environmental Conditions for Microscreen

In order to have vigorous and uniform growth of the plants in the microscreen, suitable environmental conditions are required. Among the important conditions are temperature, photoperiod, quantity of light, humidity and CO<sub>2</sub> concentration. Temperature, photoperiod and light level were not systematically studied; the following

values were found to be suitable based on prior experience with one or more of the selected species:

5                    temperature: 25-28 °C  
                    photoperiod: 14-16 h  
                    light level: 45 - 80  $\mu\text{mol}/\text{m}^2/\text{s}$  PAR.

10                   It was found that humidity surrounding the microtiter plates must be kept high enough to prevent the evaporation of water from the agar in the microtiter wells. A humidity level between 80% and 100% was suitable.

                    An important environmental parameter affecting both plant vigor and uniformity was found to be the  $\text{CO}_2$  concentration in the atmosphere surrounding the microtiter plate. Preliminary experiments with 68 species indicated that 58% showed improved growth when the atmosphere surrounding the plates was supplemented to about  
15                   10,000 ppm  $\text{CO}_2$  compared to growth at ambient  $\text{CO}_2$  concentration.

                    To further document this effect and to determine more precisely the optimal  $\text{CO}_2$  levels, microscreen plates were incubated in atmospheres containing varying amounts of  $\text{CO}_2$ . Six identical growth chambers were constructed that allowed the level of  $\text{CO}_2$  that surrounded the microtiter dishes to be controlled. These chambers were constructed of  
20                   clear plexiglass and were sealed, except for inlet and outlet ports, when the lids were attached. Flow meters and valves were used to mix pure  $\text{CO}_2$  with air to produce gas mixtures containing air (21% oxygen, 79% nitrogen) plus varying concentrations of  $\text{CO}_2$ . These gas mixtures were continuously passed through the plexiglass chambers to ensure a constant  $\text{CO}_2$  environment. Sampling ports built into the chambers were used  
25                   to periodically remove 1 mL gas samples. The  $\text{CO}_2$  content of these samples was determined using a gas chromatograph with known  $\text{CO}_2$  standards for calibration. Total flow rate through these 34.3 x 14.6 x 36.8 cm (18,429  $\text{cm}^3$  total volume) chambers was between 4250 and 9440  $\text{cm}^3/\text{min}$ . Gas mixtures were humidified by bubbling through water baths within the chambers, resulting in internal humidities ranging from 75 to 95%  
30                   relative humidity. Temperature was maintained between 25 °C and 29 °C. Light levels were between 45 and 66  $\mu\text{mol}/\text{m}^2/\text{s}$  PAR and the photoperiod was 14 h. The light, temperature and humidity levels were similar in all 6 chambers.

                    Microtiter dishes containing no herbicides were prepared as described, and placed into the plexiglass chambers for 12 days (7 days for crabgrass). At that time, the dishes  
35                   were removed and assayed for chlorophyll content as described below. Quantitative results are expressed as the mean and coefficient of variability of the chlorophyll content per seedling for a set of 32-48 plants of a single species in a single  $\text{CO}_2$  concentration. The results of one experiment are shown in Figure 1. Other, independent experiments (not shown) confirmed the conclusions of this experiment.

Figures 1A-1F show the effects of varying concentrations of CO<sub>2</sub> surrounding the microtiter dishes on the growth of seven different species (*Nicotania tobacum*, *Browallia speciosa*, *Oxalis stricta*, *Petunia hybrida*, *Coleus blumei*, *Digitaria sanguinalis*, and *Arabidopsis thaliana*) used in the microscreen. In all cases, the lowest CO<sub>2</sub> concentration tested is that derived from ambient air without supplemental CO<sub>2</sub>. (Note that the amount of CO<sub>2</sub> in ambient air varies, depending on, for example, the season and weather; in this experiment ambient CO<sub>2</sub> concentration was 550 ppm.)

In order for the microscreen to be useful, growth of plants used in the assay needs to be both vigorous and uniform. Because of this dual requirement, both the mean size of the plants and the variability between plants (coefficient of variation) are plotted in Figures 1A-1F. In general, the mean and the coefficient of variation are inversely related to each other such that conditions which give small plants generally also have highly variable plant sizes and conditions which give vigorous plants generally have lower variability.

Of the seven species tested, most show a strong dependence of growth on CO<sub>2</sub> concentration. Thus, *Arabidopsis thaliana*, *Browallia speciosa*, *Coleus blumei*, *Nicotania tobacum* and *Petunia hybrida* show less than optimum growth at ambient CO<sub>2</sub> and much improved growth as the CO<sub>2</sub> level is increased. Improved growth for *Arabidopsis thaliana*, *Petunia hybrida*, *Browallia speciosa*, *Coleus blumei* and *Nicotania tobacum*, as determined by increased chlorophyll content and reduced coefficient of variation, was observed at 2200 ppm CO<sub>2</sub>, and optimum growth was observed between 5000 and 10,000 ppm CO<sub>2</sub>. At higher CO<sub>2</sub> concentrations, growth of most of these 5 species was partially inhibited. The two other species tested either showed no strong effect of CO<sub>2</sub> level on growth (*Digitaria sanguinalis*) or an inconsistent response from one experiment to the next (*Oxalis stricta*). Thus, for 5 of the 7 species tested, supplemental CO<sub>2</sub> ranging from 5000-20,000 ppm, preferably about 10,000 ppm, was demonstrated to produce plants which were sufficiently vigorous and uniform for the microscreen. For the other 2 plants tested, supplemental CO<sub>2</sub> was not required, but it was not detrimental to their growth.

It is believed that the improvement in growth of the plants by the CO<sub>2</sub> supplementation in the atmosphere surrounding the microtiter plates is caused by a resulting increase in the CO<sub>2</sub> in the micro-environment within the wells of the microtiter plate. Poor growth of many of the plants in the absence of elevated CO<sub>2</sub> may be caused by a decrease in the CO<sub>2</sub> level inside the microtiter dishes. Although these dishes are not sealed, their lids do fit relatively tightly, thus producing a partial barrier to diffusion of gases in and out of the dishes. If the plants take up CO<sub>2</sub> by photosynthesis at a fast enough rate inside the dishes, the level of CO<sub>2</sub> may fall below the level necessary to support an optimum level of photosynthesis. By placing the dishes in elevated CO<sub>2</sub>

atmospheres, there is a higher initial level of CO<sub>2</sub> inside the plates to support photosynthetic growth. More importantly, as the CO<sub>2</sub> level inside the plates is reduced by photosynthesis, the rate of diffusion of CO<sub>2</sub> into the plates will be increased because of the larger difference in CO<sub>2</sub> levels between the external and internal atmospheres.

5        On the other hand, it is possible that the improvement caused by elevated CO<sub>2</sub> atmospheres is due to other effects of the CO<sub>2</sub>. Possible mechanisms for this effect could be the effect of CO<sub>2</sub> on ethylene or other hormone systems or the effect of CO<sub>2</sub> on the pH of the media. In any case, it is clear that elevated CO<sub>2</sub> levels are important for obtaining vigorous and uniform growth of most species of plants in the microscreen  
10    assay.

      It is apparent that the whole plant herbicide microscreen requires specialized incubation conditions that cannot be achieved using a standard laboratory incubator. Most of the plants favor elevated CO<sub>2</sub> concentrations (5000-20,000 ppm) which can be achieved by installing a CO<sub>2</sub> monitor wherein CO<sub>2</sub> levels can be carefully controlled.  
15    High relative humidity (85-90%) is also important so that the agarose in the microwells does not dry out before the end of the 14 day growth period. Most standard incubators cannot reach this level of humidity. In addition, most standard incubators have lights mounted on the bottom of each shelf, a configuration that causes the formation of a condensate on the lids of the microtiter plates. Heat from the lights causes water to  
20    evaporate and then condense on the cooler lids. As a result, plants cannot be rated for injury without first removing the condensate, because the condensate distorts the view into the plates. This problem can be solved by insulating the space between the lights and the shelf. Percival Scientific Inc., Model CU-32L tissue culture chamber offers a suitable configuration. Moreover, the lights in this incubator are arranged in a "tic tac  
25    toe" pattern that results in more uniform lighting in the incubator. In addition, air is circulated across each shelf to give more even temperature and humidity control. This incubator can be modified by installing a CO<sub>2</sub> monitor, as well as a modified humidifier which can achieve humidities up to 92%.

#### Seed Germination

30        Uniform seed germination is required in order to maximize the utility of a whole plant herbicide microscreen assay. Accordingly, seed germination was evaluated under a variety of seed pretreatment protocols. *Browallia speciosa*, *Coleus blumei*, *Digitalis purpurea*, *Digitaria sanguinalis*, *Nicotiana tobacum*, *Oxalis stricta* and *Petunia hybrida* seeds were treated in five different ways prior to planting (ethanol, acid, bleach, UV  
35    sterilization and cold). A small amount of each seed was placed in a 1.5 mL micro centrifuge tube and treated with ethanol, acid or bleach as described below. One milliliter of 0.3 M ethanol in deionized water was added to seeds and seeds were soaked for 24 h (Taylorson, R. B. *Weed Science* (1989) 37, 93-97). One milliliter of 6 M

sulfuric acid was added to seeds and the tube was shaken for 1 min. Then the seeds were soaked for 15 min and rinsed 5 times with deionized water (Brecke, B. J. and Duke, W. B. *Weed Science* (1980) 28(6), 683-695). One milliliter of 25% bleach (125 mL Clorox, 375 mL deionized water, 10 drops of Triton™ X-100 (Sigma Chemical Co., St. Louis, MO) was added to seeds and the tube was shaken for 2 min. The seeds were soaked for 7 min, then rinsed 5 times with deionized water. For all seed treatments, the wet seeds were transferred to a petri dish containing filter paper and the seeds were allowed to dry overnight.

Seeds were also UV sterilized by placing seeds on top of a UV light box in a sterile hood which was also illuminated with a UV lamp from above. Seeds were exposed to the UV lights for 16 h. For cold treatment, seeds were planted in agarose in 96-well microtiter plates and placed in a refrigerator at 4°C for 24 h.

Plates were prepared as described above with one microtiter plate containing seed from one plant species which received one seed treatment. Plants were visually evaluated at 14 DAP. The results for each tested plant species are presented below.

*Browallia*: *Browallia* had 100% germination for all treatments. The acid treatment was unacceptable because the plants were very uneven in growth and had poor growth in general. The UV and bleach treatments both had a few malformed plants, so these treatments were of questionable use. Plants under cold treatment were slightly smaller than controls but otherwise these plants were similar to controls. The ethanol treated plants were also similar to controls but slightly less uniform. Controls appeared to be the best plants. Therefore, no seed treatments were necessary for growth of *Browallia* in microtiter plates.

*Coleus*: The germination rate for all treatments of *Coleus* was excellent (98-100%). Control (untreated) *Coleus* looked excellent and grew very uniformly with a good green color. Bleach and ethanol treatments looked similar to controls. The acid treatment was somewhat uneven and showed some bleaching. The UV treated plants were slightly less uniform than controls and the cold treated plants were smaller than controls. Control plants looked best; no seed treatments were necessary for growth of *Coleus* in microtiter plates.

*Digitalis*: The bleach treatment was best for *Digitalis* with 100% germination compared to 83% germination with controls. Bleach treated plants were also more uniform than controls. With all treatments however, under the optimized conditions of this test, the *Digitalis* plants were too large for the plates and were therefore eliminated from further consideration.

*Digitaria*: The germination rate of *Digitaria* varied depending on type of seed treatment. Control (untreated) *Digitaria* had 96% germination, were fairly

uniform in growth and had a pale green color. Bleach treated plants had 85% germination, were stunted and were less uniform in growth than control (untreated plants). The bleach treatment was unacceptable. The acid treated plants had 92% germination and were slightly less uniform in growth than controls. UV treated plants looked similar to controls and had 96% germination. Cold treated plants were slightly larger than controls and had 94% germination. Ethanol treated plants had the highest germination rate (98%) and looked similar to controls except for some slight bleaching. Overall the controls were the best looking plants. Therefore, no seed treatments were necessary for growth of crabgrass in microtiter plates.

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*Nicotiana*: For all treatments, the germination rate for *Nicotiana* was 96-98% and no differences were observed in growth/uniformity from the control (untreated) *Nicotiana*. The control *Nicotiana* was very uniform with 4 leaves and very erect plants with a deep green color. No seed treatments were necessary for growth of *Nicotiana* in microtiter plates.

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*Oxalis*: The acid treatment was unacceptable for *Oxalis* since no seeds germinated. The UV and bleach treated plants showed some bleaching and were more uneven in growth compared to controls, so these treatments were eliminated. The germination rate for ethanol treated plants was slightly lower than controls, 88% for ethanol compared to 92% for controls and the plants were more uneven in growth. The cold treated plants, however had a slightly higher germination rate than controls, 94 and 92%, respectively and were slightly more uniform in growth. The cold treatment was selected as the method of choice for growing *Oxalis* in microtiter plates.

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*Petunia*: The germination rate was excellent (98-100%) for all treatments of *Petunia*, but there were differences in growth uniformity and appearance. The control (untreated) *Petunia* were pale green and uneven in growth and were not ideal for use in the microscreen. The cold treated plants were very uneven in growth and were found to be unacceptable. Bleach treated *Petunia* showed a bleached appearance and were also not acceptable. The UV and ethanol treated plants looked slightly better than controls (more uniform in growth). The acid treated *Petunia* had 100% germination and the plants were larger, fairly uniform in growth and a good color. The acid treatment was selected as the best method for growing *Petunia* in microtiter plates.

35 Assay Automation

Solubilization of compounds: Tests were conducted to determine the maximum amount of DMSO each plant species would tolerate. DMSO was selected as the universal solvent for solubilizing compounds into aqueous media because it is polar, less



volatile than other organic solvents and is an excellent solvent for most smaller organic molecules. In order to determine the maximum concentration that each species could tolerate, DMSO was added to 1/2 x MS media and mixed with agarose to yield 0, 0.1, 0.3, 0.6, and 1.0% DMSO solutions. One seed was planted in each well with 32 wells per species. Injury was evaluated relative to control (untreated) plants. The following ten species were tested: *Arabidopsis thaliana*, *Browallia speciosa*, *Coleus blumei*, *Digitaria sanguinalis*, *Eragrostis cymbula*, *Inula ensifolia*, *Nicotiana glauca*, *Oxalis stricta*, *Panicum coloratum* and *Petunia hybrida*. Plants were evaluated for injury 14 DAP. The following observations were made:

- 10 *Arabidopsis*: No injury was observed at the highest concentration of DMSO tested (1.0%).
- Browallia*: No injury was observed at the highest concentration of DMSO tested (1.0%).
- Coleus*: No injury was observed at the highest concentration of DMSO tested (1.0%).
- 15 *Digitaria*: No injury was observed at 0.1% DMSO. At 0.3 to 1.0% DMSO there was slightly more bronzing of the newest leaf.
- Eragrostis*: No injury was observed up to 0.3% DMSO, but at 0.6 and 1.0% DMSO there was bronzing/burning of leaf tips.
- 20 *Inula*: DMSO improved germination, growth uniformity and greenness. Plants treated at 1.0% DMSO appeared to be the most healthy.
- Nicotiana*: No injury was observed at the highest concentration of DMSO tested (1.0%).
- Oxalis*: No injury was observed up to 0.3% DMSO. The plants had a poor appearance at 0.6% DMSO and germination dropped by 45% at 1% DMSO.
- 25 *Panicum*: No injury was observed up to 0.3% DMSO, but at 0.6 and 1.0% DMSO, the plants were slightly chlorotic at the leaf tips.
- Petunia*: No injury was observed up to 0.3% DMSO. At 0.6 and 1.0% DMSO, slight injury was observed.
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*Digitaria* was most sensitive to DMSO and thus dictated the concentration to be used for solubilization of compounds. Based on results with *Digitaria*, the concentration of DMSO used was 0.1%.

Automation of Plate Preparation: Five milligrams of each compound were weighed into deep well microtiter plates, 88 compounds per plate with the first column empty

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- (column 1) for controls. Using a Tomtec Quadra-96™ pipettor, 500 uL of DMSO were pipetted into each well to prepare a 10,000 ppm compound solution. A lid was placed on each plate, sealing each well and each plate was tumbled for 10 min to solubilize the compounds. Each plate was centrifuged for 15 seconds to remove DMSO from the lids.
- 5 These plates were the mother plates. Daughter plates were prepared from mother plates by pipetting 425 uL of 1/2x MS media into each well of the daughter plate (1.2 mL deep well plate) using the Quadra-96™. This step was repeated. Then 147 uL of 1/2x MS media was drawn into pipette tips plus 3 uL of DMSO from the mother plate and dispensed into daughter plate with mixing. Using the Quadra-96™, treatment plates were
- 10 prepared by drawing 250 uL from the daughter plate (30 ppm compound solution) and dispensing 33 uL into each well of 4 standard microtiter plates. Using the Quadra-96™, 400 uL 0.6% agarose were drawn into pipette tips and 66 uL were dispensed into each well of the 4 treatment plates (standard microtiter plates) yielding a 10 ppm compound solution in 0.4% agarose. No additional mixing steps were necessary for mixing of the
- 15 agarose and 1/2x MS media/compound solutions. A total of 16 treatment plates were prepared from each mother plate to accommodate 7 species (1 species per plate) and duplicate tests (2 plates were discarded).

#### Automation of Seeding

- One seed of each species is planted manually in each well using a forceps. In
- 20 addition, several methods for automation of seed dispensing are possible. Several methods of automating the seeding have been tested with the goal of placing one seed in each well of a 96-well microtiter plate.

- A template seeder was designed by Berry Seeder Co. (Elizabeth City, NC) for seeding into 96-well microtiter plates. This seeder has stainless steel plates with 96 holes
- 25 which are in alignment with the microtiter plate wells. There were 4 stainless steel plates, each with different diameter holes to accommodate the different sized seeds. Each stainless steel plate is attached to a plexiglass box containing a vacuum line. High pressure air was run into a separate valve in the box and opening the valve resulted in vibration of the stainless steel plate. Seeds were placed on the stainless steel plates with
- 30 the vacuum running and the high pressure air valve opened to vibrate excess seeds out of each hole. Experiments were run with *Arabidopsis thaliana*, *Browallia speciosa*, *Coleus blumei*, *Digitalis purpurea*, *Digitaria sanguinalis*, *Nicotiana tobacum* and *Petunia hybrida*. The Berry template seeder worked well with *Browallia* and *Coleus* seeds with 90-95% singulation. These seeds are quite round and were the largest seeds tested
- 35 (0.040" diameter for *Coleus* and 0.035" diameter for *Browallia*). *Digitaria*, which is an elongated seed, could not be singulated because it would not stick in the holes (holes were too small). With *Petunia* and *Digitalis* seeds, the holes in the stainless steel plates were either too large (seeds were pulled through the holes) or too small (seeds would

not stick). *Arabidopsis* seeds had multiple seeds in many of the holes. *Nicotiana* seeds had 26-45% singulation with the remainder being either multiple seeds or no seeds (seeds would be pulled through the holes). Although the Berry template seeder had high singulation of the *Coleus* and *Browallia* seeds, the seeder is quite temperamental. It takes some "tweaking" to attain this level of singulation. This seeder did not work well with the other seeds. Based on these tests, this seeder would not work for automation of seeding.

A needle seeder (Seed-Air-Matic™, KW Engineering Pty. Ltd., Queensland, Australia) for seeding into plug trays has been tested to determine the feasibility of using with the following seeds: *Arabidopsis*, *Browallia*, *Coleus*, *Digitaria*, *Nicotiana*, *Oxalis* and *Petunia*. This seeder has 8-20 syringe needles of varying bore sizes that were connected to vacuum. Seeds were placed in a hopper that vibrates. The needles dipped into the hopper, picked up individual seeds and dropped the seeds into guide tubes, which in turn released into plug trays using a short burst of high pressure air. A row of seeds was picked up and dropped in 1-4 seconds. The KW Engineering seeder was used to test singulation of *Arabidopsis*, *Browallia*, *Coleus*, *Digitaria*, *Nicotiana*, *Oxalis* and *Petunia* seeds into 96-well microtiter plates. This seeder has been modified to seed into microtiter plate format. *Browallia*, *Coleus*, *Digitaria*, *Nicotiana*, *Oxalis* and *Petunia* seeds had 90-96% singulation using a single needle. *Arabidopsis* singulation rate was about 83-85%. Individual vacuum regulators have been installed to achieve these levels of singulation with multiple needles (8-12). At these singulation rates, missed or multiple seeded wells can be corrected manually.

Batching Systems (Owings, MD) uses a vibratory bowl to singulate parts (nuts, bolts, game pieces, etc.) and an optical eye to count the parts. For use as a seeder specifically for seeding into 96- or 48-well microtiter plates, seeds would be placed in a vibratory bowl and would "walk" up a ramp. At the end of the ramp, the width would be adjusted so that only one seed at a time could fit on the ramp. The seeds would drop down and be counted by a dual-view optics system. A microtiter plate would be placed on an X-Y table to singulate seeds.

A preferred method of seeding comprises a combination of elements making up a seed dispensing system that features a seed pick and place mechanism for a plurality of containers, a seed placement monitoring device for a plurality of containers, and a control system to inform the operator of the location of potentially misplaced seeds or to provide feedback for automatic re-seeding of any missed containers. Alternatively, a discrete correction station may be a component of the system. A preferred embodiment includes in the pick and place mechanism the feature of individual control of vacuum to individual seed holding tubes (or needles). This individual control of vacuum combined with the seed pickup tube design improves the reliability for single seed pickup.

Additional features of the preferred method include the proper positioning of tip of each seed holding tube during seed pickup and during seed deposition, and providing in each container a substance that when contacted with the seed will inhibit or prevent further movement of the seed within the container and inhibit or prevent the seed from being expelled from the container due to air currents and/or static charge.

Figure 4 shows the schematics of the basic elements of an automated system for placing seeds into wells of a plurality of microtiter plates, monitoring the accuracy of placement, and correcting for errors. The system will also work to place insect eggs into microwells so as to enable an automated insecticide micro-assay, although the system will be described in the context of handling seeds for an automated herbicide micro-assay. A control device 20 is connected to a seed handling and monitoring device 22 and a seed correcting station 24. The seed handling and monitoring device consists of a base 26 covered by an enclosure 28 which may be supplied with positive pressure filtered air by air supply device 30 or may be provided with negative pressure to control volatile organic compounds. Access door 32 allows access for placing seeds in a seed supply station 34, and access door 36 allows access for loading and unloading a plate carrier 38 with a plurality of microtiter plates, such as plate 40. Within the enclosure is a transport mechanism 42 for transporting an overhead module 46 that contains a monitoring camera 48, a seed holder 50 and a plate identification reader 52.

Figure 5A shows an detailed view of the seed handling and monitoring device 22, and Figure 5B shows a break away of a portion of the seed handling system. The base 26 supports the removable plate carrier 38 that is shown with a plurality (4) of microtiter plates 40, 54, 56, and 58 in predetermined locations. The microtiter plates are of the size that contain 96 wells, such as well 59 in plate 58, in a 12X8 array and each plate has a bar code label, such as label 60 on plate 58, preferably on the top surface of the plate. Plates having different dimensions and different numbers and configurations of wells can also be used by making appropriate changes in the seed holder 50. Within each well is a premeasured volume (not shown) of a compound to be tested and a plant growth medium, which can also contain agar. There may be a different compound in each well and several control wells in each plate. Different plates may contain the same compounds to check reproducibility. The carrier can be configured to hold more or less than the illustrated 4 plates. The carrier may be placed on the base either manually, as described via door 36, after removing a lid (not shown) from each plate, or the carrier may be placed automatically from an autofeeder that may hold up to 15 carriers and may include a lid handling feature.

The transport mechanism 42 consists of an overhead X-Y positioning system for overhead module 46, the mechanism comprising a first carriage 62 that runs on rails 64 and 66 for positioning the module in the X-direction indicated by arrows 68; and a

second carriage 70 that runs on rails 72 and 74 for positioning the module in the Y-direction indicated by arrows 76. Attached to the carriage 70 is the overhead module 46 that comprises actuator 78, seed holder 50, monitoring camera 48, plate identification reader 52, and seed catcher 80. Actuator 78 mounted on module 46 is for positioning the seed holder 50 in the Z-direction indicated by arrows 82. Elements making up module 46 may be attached to a module frame 84 as shown, or they may be attached directly to carriage 70. The seed holder 50 (Figure 5B) comprises a plurality of seed pickup tubes, such as tube 79 and tube 81 that extend from tube block 83. The seed catcher 80 is attached to actuator 78 and comprises two moveable arms 86 and 88 that support angled bars 90 and 92 respectively that are underneath the ends of the plurality of seed pickup tubes when they are in the upper Z-direction. Actuators 94 and 96 control the motion of arms 86 and 88 respectively.

The seed supply station 34 consists of a seed trough 98 for holding a supply of seeds, a frame 100 resiliently supporting the trough, and a vibrator 102 attached to the trough for vibrating it to cause the seeds to be fluidized or air-borne in the trough. The trough may contain dividers, such as divider 104 to keep the seeds contained in individual compartments in line with individual seed pickup tubes during pickup by seed holder 50. The trough and dividers are preferably made from a conductive material, such as stainless steel or a filled conductive polymer, that can be grounded to prevent static electricity buildup. The seed supply station may be located in a central position to minimize travel distance to all the wells.

In Figure 5A, the transport mechanism 42 is shown in a position to permit insertion of seeds in a row of wells, such as well 59 in row 106 in plate 58. In this position, plate identification reader 52, also known as a bar code reader, has passed over bar code 60 and identified plate 58 to the control device 20 (Figure 4). There are position encoders (not shown) on the X and Y carriages so the controller 20 can confirm which row of wells the seed holder is positioned over. The seed catcher 80 is in a closed position as shown to catch any seeds that may be dislodged during transport of the seeds from the supply station 34 to the row of wells 106. If a seed were to be dislodged, it would fall into one of the angled bars 90 or 92 instead of landing in one of the wells being crossed during transport, which would result in a seeding error that may not be detected. Periodically, the bars 90 and 92 would be emptied of fallen seeds. Before the seed holder can proceed in the Z-direction to place the seeds in a row of wells, the seed catcher must be in an open position with the arms 86 and 88 moved in the direction of arrows 108 and 110 respectively. This will allow the tube block 83 to pass between bar 90 and bar 92 so that the tips of tubes, such as 79 and 81 can enter the wells, such as 59, in row 106 and deposit in each well of row 106 a seed held by each tube. After deposit of the seeds, the tube block would be retracted in the Z-direction and the

transport mechanism 42 would move to place camera 48 over row 106 so each well in row 106 could be monitored for the presence or absence of seeds. The camera 48 is positioned far enough above the wells that its field of view includes all 8 wells in row 106 and the wells are shallow enough at that position so the image of the outermost wells still enables the camera to see the bottom of the well where the seed is deposited. By "bottom of the well" is meant the top surface of the plant growth medium containing compound that is placed in the well and is the surface on which the seed will rest. Typically, in a 10 mm deep well there may be about a 4 mm deep layer of plant growth medium containing compound so the "bottom of the well" is about 6 mm down from the top of the well.

Seed holder 50 is shown with a linear array of 8 tubes, such as 79 and 81, in block 83. This could alternatively be a rectangular array of tubes, such as a 4X2 or 4X4 array, or any other similar arrangement. A more compact array may have an advantage to make monitoring of the wells by camera 48 more reliable by minimizing any parallax optical problems or allowing the camera to be located closer to the wells.

Referring to Figure 6, which is an enlarged section view of tube 79, the tube has a relatively large outer diameter 112 of about 1-4 mm, preferably about 3.0 mm to resist bending and enable accurate positioning in the well. Throughout most of the length of tube 79 there is an enlarged bore 114 of 0.5-3 mm, preferably of about 1.5 mm that terminates near the tip 116. The tip may have a chamfer 118 to present a small flat end 120 to the seeds and deflect adjacent seeds, in motion in a fluidized bed of seeds, away from the single one held at the flat end. A relatively small diameter hole 122 extends from the end of bore 114 through the tip 116 for fluid communication with a single seed. The size of the hole is approximately proportional to the size of the seed. Conveniently, the hole will be circular in cross-section and the diameter of the hole may be smaller than smallest dimension of the seed. For unusually small seeds, this hole 122 may have a diameter of only 0.05 mm. Bore 114 is in communication with a source of air that alternately may be under vacuum or pressure. The pressure can be useful to clear any debris from the hole 122 and to dislodge the seed. To eliminate problems with static electricity near very small lightweight seeds, tube 79, which is representative of all tubes, is made of a conductive material which is corrosion-resistant, such as 304 stainless steel, and is grounded via the block 83, which is also conductive, and a grounding cable 124 (Figure 5A) or other suitable grounding means.

The camera 48 is arranged above the wells at a distance sufficient to obtain a clear high resolution view of the bottom of the wells. Figure 7 illustrates an enlarged perspective view of what the camera sees looking at the row 106 of wells. Circle 126 represents the top of well 59, circle 128 represents the bottom of the well that is a slightly smaller diameter than the top circle 126. Typically, the top surface of plant

growth medium containing compound mix will define the visual bottom of the well. Part of the very edge of bottom circle 128 may be partially blocked from view by top circle 126 as is shown at 130. This is due to parallax which is an apparent shift in position of the bottom circle relative to the top circle due to the actual line of sight from the camera center 131 to the well, which is different for each well. Surface 132 represents the sidewall of well 59 that connects the top circle to the bottom circle. Dashed circle 134 represents the target circle for a seed, such as seed 135, placed at the bottom of the well. It is a smaller diameter circle than bottom circle 128 so the seed will be spaced away from the sidewall surface 132. In other wells, such as well 136 near the center of row 106, there may not be any blocking of the bottom circle by the top circle. In the view of Figure 7, the camera can see the entire area of the target circle for all wells. This is important so the camera can notify the control system if the seeds have been successfully placed in the target circle of each well. Based on the camera view, the control system can make several determinations about each seeded well:

- 1) one seed in the target circle only;
- 2) no seed in the target circle and no seed in the well;
- 3) no seed in the target circle and one seed in the well;
- 4) more than one seed in the well, which may be in or out of the target circle.

A seed that falls at the corner where the bottom circle meets the sidewall may not be seen due to the poor contrast at the corner; and a seed that falls in a blocked area like 130 may not be seen. In such circumstances, there may be a small degree of uncertainty about the determinations. It is expected that such circumstances will be rare due to the accuracy and reliability of the seed placement system. Ideally, such uncertainty is avoided by use of multiple cameras looking at a row or a single camera indexing over two viewing positions, each with fewer wells, to eliminate any parallax problem. Using shallower microtiter wells may also eliminate any parallax problem. The microtiter plates are backlighted to give good lighting and contrast at the bottom of the well for the camera.

If determination #1 is made for all just-seeded wells, the control system can tell the transport mechanism to go get seeds for the next row of wells. If determination #2 is made for any well of the just-seeded wells, the control system can tell the transport mechanism to go get a seed for the particular well that is missing a seed in the target area. If determination #2 persists for the same cell after an attempted correction, the control system can create an error data file to alert the operator that a #2 defect exists for this particular well. If determination #3 is made for any well of the just-seeded wells, the control system can create an error data file to alert the operator that a defect #3 exists for a particular well. If determination #4 is made for any well of the just-seeded wells, the control system can create an error data file to alert the operator that a defect

#4 exists for a particular well. The error data file can be used to alert the operator to make manual corrections. In an alternate embodiment, the seed correcting station 24, referring to Figures 4 and 8, is a manually operated/machine assisted station where an operator can place a microtiter plate and examine and correct for determinations #2, 3, and 4 as identified by the camera and control system. It consists of a plate holder 138 that supports a barcoded microtiter plate 139 that a barcode scanner 144 can read. This allows the control device 20 to identify a particular plate. The microtiter plate 139 is over an LED array 140 located within the holder as seen in the cutaway view of Figure 8. The LED array 140 consists of an illuminating LED positioned under each well of the microtiter plate, which in the case shown would be an 8X12 array of 96 LEDs. The control device 20 can independently illuminate particular LEDs by accessing error data files to locate the wells with the #2, 3, and 4 determinations based on the earlier examination of that plate by the monitoring camera 48. The operator can then take the corrective action for each illuminated well. Discriminations between determinations can be made if desired by the manner in which the LED is illuminated, i.e., continuously illuminated, blinking slowly, blinking quickly, etc. The station 24 also has an input switch 142 to permit communication with the control device 20 to turn off selected LEDs, and indicate manual correction has occurred.

Part of the seed holder 50 is a pneumatic system for controlling the seed pickup and drop off function as seen referring to Figures 9 and 6. Referring to Figure 9, each seed pickup tube, such as tube 79 or 81 in tube block 83, is in fluid communication via conduits 146 and 148, respectively, with pneumatic system 150. This system will be described referring to tube 79 and is understood to be the same for other seed pickup tubes. Conduit 146 is connected to a solenoid operated, three-way, selector valve 152 which is connected to a vacuum generator 154 and a pressure manifold 156. The vacuum generator produces vacuum by a venturi effect and is connected to a pressure regulator 158 and a solenoid operated, two way, selector valve 160, and a pressure manifold 162. The pressure manifold 162 is connected to a source of pressure 164. Pressure manifold 156 is connected to a pressure regulator 166 which is connected to pressure manifold 162. The system allows individual vacuum adjustment to each seed pickup tube for the purpose of 1) adjusting to individual tolerances in the small seed pickup hole, such as hole 122 in tube 79 (Figure 6), and 2) individually turning "on" the vacuum in one or more tubes while it remains "off" in the remaining tubes. The individual vacuum level, or flow, for a tube is individually triggered by actuating the selector valve 160 to direct pressure to the vacuum generator, and then adjusting the pressure regulator 158 to achieve the desired vacuum level. The individual adjustment would be done by trial and error by observing the seed pickup performance for that tube. Alternatively, for automated monitoring of the pneumatic system, there is a pressure



transducer 168 between the regulator 158 and vacuum generator 154, and a pressure transducer 170 between pressure regulator 166 and pressure manifold 156. The transducer, such as 168 for each seed pickup tube, and transducer 170 are connected electrically to the control device 20 for continuous monitoring.

5        During continuous automated operation, the seed handling and monitoring system works as follows referring to Figures 4, 5A, 5B, 6, 8, and 9. Beginning at the position shown in Figure 5A, the operator has already loaded the seeds in the seed supply station 34 and has loaded carrier 38 with microtiter plates 40, 54, 56 and 58 that are prepared with plant growth medium and a different compound for testing in each well,  
10        such as well 59. The operator removes the lids from the plates and places the carrier 38 in the enclosure 28 and makes sure the air supply/suction device 30 is providing a steady low flow of filtered air/negative pressure to the enclosure. The operator closes the doors 32 and 36. This minimizes the chance of airborne contamination and controls any air currents that may tend to dislodge seeds during handling. The seed holder 50, as  
15        shown in Figure 5B, has already successfully picked up seeds from seed supply station 34 and is positioned over row 106 in preparation for depositing seeds in the wells of row 106.

Automated operation, illustrating reseeding, proceeds as follows:

- 20        - valves, such as 160 and 152, remain actuated to maintain vacuum (produced by the vacuum generator 154) and hold a single seed at the tips, such as 116, of all seed holder tubes, such as 79;
- the seed catcher 80 is actuated to move the bars 90 and 92 away from the tips such as 116 of seed holder tubes such as 79;
- actuator 78 is energized to lower the tube block 83 toward the microtiter plate 58  
25        until the tips such as 116 of the tubes such as 79 are inside the wells such as 59 of row 106, which typically places the tips such as 116 about 4 mm away from the bottom of the wells;
- valves, such as 152, are actuated to shift the vacuum "off" and pressure (set at regulator 166) "on" to all the seed holder tubes such as 79 to release the seeds and  
30        project them from the tips such as 116 and into each well in row 106, and onto the sticky surface forming the bottom of the wells;
- actuator 78 is energized to raise the tube block 83 back to the upper position and valves, such as 152, are actuated to shift the pressure "off" and the vacuum "on" to tubes such as 79;
- 35        - the transport mechanism 42 moves the overhead module 46 to position the camera 48 over row 106 where the seeds were just placed. The camera "reads" the wells and confirms that a single seed has been properly placed in all wells in row 106, except

one, and discovers there is no seed in the target and no seed in the bottom of one well in the row, and the control device 20 notes this in an error data file;

- the control device 20 turns "off" the two-way valves 160 to all seed holder tubes except the seed holder tube for the one well that is missing a seed;

5       - the control device 20 commands the transport mechanism 42 to align the overhead module 46 over the seed supply station 34;

- the actuator 78 is energized to lower the tube block 83 until the tips such as 116 are in the fluidized bed of seeds in the seed trough 98 (for some seeds, the tip is best located just above the upper surface of the fluidized seeds, for other seeds, the tip is best located just at the surface of the fluidized seeds and for some others the tip is located just below the surface of the fluidized seeds);

- the actuator 78 dwells from about 0.01-10 seconds while vacuum is applied to only the one tube to engage a single seed;

- the actuator is energized to raise the tube block 83 back to the upper position;

15       - the seed catcher 80 is actuated to move the bars 90 and 92 toward the tips such as 116 of seed holder tubes such as 79 to put the bars in position to catch any seeds falling from the ends of the tubes;

- the transport mechanism 42 moves the overhead module 46 over row 106 in plate 58 with the tube block aligned over the row 106;

20       - the seed catcher 80 is actuated to move the bars 90 and 92 away from the tips such as 116 of seed holder tubes such as 79;

- actuator 78 is energized to lower the tube block 83 toward the microtiter plate 58 until the tips such as 116 of the tubes such as 79 are inside the wells such as 59 of row 106;

25       - the appropriate valve, such as 152, is actuated to shift the vacuum "off" and pressure "on" to the appropriate seed holder tube 79 to release the seed from the one tube and project it from the tip 116 of that tube and into the well that was previously missing a seed;

30       - actuator 78 is energized to raise the tube block 83 back to the upper position and the appropriate valve, such as 152, is actuated to shift the pressure "off" and the vacuum "on" to the appropriate tube such as 79;

- the transport mechanism 42 moves the overhead module 46 to position the camera 48 over row 106 where the missing seed was just placed. The camera "reads" the wells and discovers there are now single seeds in the target for all wells;

35       - the control device 20 turns "on" the two-way valves, such as 160, to all seed holder tubes that had previously been turned "off";

- the control device 20 commands the transport mechanism 42 to align the overhead module 46 over the seed supply station 34;

- after picking up seeds on all tubes such as 79, the control device will command the transport mechanism to position the overhead module over the next row of wells in the microtiter plate and the process will continue until all wells on all plates in the carrier have at least one seed;

- 5       - the control device 20 will alert the operator to remove the carriage from the enclosure and notifies the operator about plates that require corrective action;
- the operator places any plate requiring corrective action in the seed correcting station 24;
- the control device identifies the plate with information from bar code scanner 144  
10 and illuminates the LED or LEDs beneath the wells that require corrective action;
- the operator takes corrective action on each well requiring it. Upon completing each plate, the operator actuates the input switch 142 to notify the control device 20 to turn off the LEDs to the corrected wells and update the control device data files to indicate manual correction to this microtiter plate is complete.

15       While the method and apparatus of the present invention is described in detail above with respect to an herbicide assay, this technology can also be used effectively for other assays such as an insecticide assay.

      When handling insect eggs with the system just described for seed handling, there are a few variations in the procedure. It may be desired to place the eggs on the inside  
20 surface of an elastomeric lid for a microtiter plate, and it may be desired to use a square well microtiter plate that has wells about 1 3/4" deep so there is adequate space for the insect larve and insect to develop. The elastomeric lids have square protrusions that extend into each square well of the microtiter plate, and each protrusion has two holes in the corners of the protrusion for entry of oxygen for the larve and insect when the lid is  
25 in place over the well. A sticky drop of larve support and growth medium is placed on the inside surface or each lid protrusion to hold the eggs in place and support the eggs until the larve hatches. Only lids are placed in the enclosure 28 on the carrier 38 with the inside surface of the lid facing upward. The seed supply station 34 would be used to supply the eggs in much the same way it is used to supply seeds. The overhead module  
30 46 would be much the same for handling and monitoring eggs as for seeds. After placement of the eggs on the lids, the lids are removed from the enclosure and manually placed on the matching microtiter plate for that lid. After hatching, the larve drops into the well and onto the surface of a mix of larve/insect support medium and test compound in the well that fills the well to a height of several millimeters. The effect of the  
35 compound on the larve/insect can be observed over a period of time.

      Figure 10A shows a partial perspective view of an inverted microtiter lid 172 and egg pickup tubes, such as tube 174, for handling insect eggs, such as 176. If there is a problem with the elastomeric lids distorting when laid free and held only by gravity, one

can force them to lay flat for the accurate positioning needed for accurate reliable egg placement, using a weighted frame 178 laid on top of the inverted lid held in place on a carrier 179 (similar to the microtiter plate carrier 38). The frame 178 includes a peripheral rim 180 and central rims 182 and 183 to hold the edges, such as lid edge 184, and central part of the lid, respectively, in a flat condition. The lid 172 has a plurality of protrusions, such as protrusion 186, that are arranged to extend into each well of a microtiter plate (not shown). Each protrusion has a hole in two corners of the protrusion, such as holes 188 and 190 in protrusion 186.

Figure 10B shown an enlarged section side view of a protrusion of a lid and an egg pickup tube. One or more eggs 176 are held on the tip 192 of tube 174 by application of vacuum to the tube. A hole (not shown) extending through tip 192, similar to the hole in seed tube 79 of Figure 6, is sized to be a slightly smaller diameter than the minor dimension of a single insect egg, which may have a slightly irregular spherical shape. Because of the irregular shape of the eggs, the vacuum flow can extend around each egg so several eggs can be picked up at one time. The number of eggs picked up can be controlled by adjusting the level of vacuum applied through the hole in tip 192. On top of protrusion 186 is a drop 194 of sticky material for holding the eggs and providing some nutrient for the eggs. When depositing the eggs on top of the protrusion 186, the tip 192 of tube 174 is lowered to within a closely spaced distance 196 of about 4 mm from the surface of the drop so the eggs will be reliably deposited onto the sticky drop when the vacuum is shut off and a pressure is applied to the hole in tip 192. After placing eggs on the top of a row of protrusions, the camera monitor 48 would be positioned over this row of protrusions and would monitor each protrusion in the row to determine if there were some eggs placed there. If there were no eggs present, the control system would tell the transport mechanism to go get eggs for the protrusion missing them, similar to the procedure followed for handling seeds.

Variations in the operation of the seed or egg handling and monitoring system is possible without departing from the basic teachings of the invention. For instance, while normally one and only one seed is desired in each well, there could be circumstances where more than one seed and more than one plant would be desired in each well, such as if the weed being evaluated were sedge, which is extremely small and is a common weed problem in rice crops. In such a circumstance, it would be most convenient to simply repeat the deposition of single seeds the appropriate number of times. Other variations of the invention include different camera monitoring strategies and the use of automated carrier handling. In addition, the carrier may be replaced with a precision indexing conveyor that will accurately move one plate at a time into a seed depositing position under a simplified transport mechanism. Plates would be automatically loaded onto the conveyor. The simplified transport mechanism would only move in the

X-direction 68 and Z-direction 82, and there would only be two possible X positions. One X position would be with the seed pickup tubes over the seed supply station and at the same position, the camera is over the depositing station monitoring the seed placement. The other X-position would be with the seed pickup tubes over the seed depositing station. The indexing conveyor would position the relevant row of wells at the depositing station. The other wells not at the depositing station would be covered under a shield so the seed catcher 80 could be eliminated.

#### Assay Scoring

Several assays were evaluated for scoring the microscreen. A visual assay was used initially and has continued to be used throughout the development of the microscreen because of its simplicity and similarity to standard greenhouse herbicide assays. This assay worked well for the microscreen and, given only several hours of training and experience, can be learned and reproducibly performed by anyone knowledgeable about plants. Several other scoring assay formats were also evaluated.

Visual Assay: Plants were scored visually. Size, morphology, and color were taken into consideration and a score of 0 to 4 was assigned. Table 4 describes the rating system that was adopted for visual assay scoring.

Table 4. System for rating plants for herbicidal injury.

Rating	% Visual Injury
0	No injury relative to controls
1	1-25% injury relative to controls
2	26-50% injury relative to controls
3	51-75% injury relative to controls
4	76-100% injury relative to controls

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Biomass Assay: Individual plants were removed from the microtiter wells, blotted briefly on paper towels to remove adhering water and then their mass was determined using an analytical balance (Mettler AE50).

Chlorophyll Assay: Other assays were also developed which, compared with the visual and biomass assays, might be more easily automated. An assay which measures chlorophyll content of the plants was developed which requires neither the removal of the plants from the microtiter plate wells nor the grinding of the plant material. This was accomplished by taking advantage of the fact that DMSO will solubilize chlorophyll from intact plant tissue (Hiscos, J. D. and Israelstam, G.F. *Can. J. Bot.* (1979) 57, 1332-1334). However, the DMSO was not able to extract chlorophyll from plant material when the DMSO was added directly to the plants and agarose in the microtiter plates. The water in the agarose (~100 uL) diluted the 200 uL of DMSO sufficiently that it could not solubilize the chlorophyll. To circumvent this problem, the water in the

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agarose and plant material was removed by lyophilization. DMSO then readily extracted the chlorophyll from the resulting dried agarose and plant material. Because of the simplicity of this assay, it is not only easily performed manually but also can be readily automated.

5        Several tests were conducted to validate the chlorophyll assay. In order to determine whether the chlorophyll assay was a good measure of overall plant growth, a set of 95 *Arabidopsis* plants which were variable in size (biomass coefficient of variation = 0.63) were removed from the microtiter plate and their fresh biomass was determined using an analytical balance. The plants were then returned to the microtiter plate wells  
10        and their chlorophyll content was measured.

Microtiter plates were frozen at -20 °C and then lyophilized. DMSO (200 uL) was added to each well and the plates were incubated in the dark at 65 °C for 1 h with gentle shaking (about 30 rpm). Plates were then cooled to room temperature in the dark. The absorbance of the DMSO solution was then measured in a Molecular Devices VMAX™  
15        Kinetic Microplate reader. The absorbance at 650 nm (near the peak of the chlorophyll absorbance spectra) was corrected for scattering by subtracting from it the absorbance at 562 or 570 nm, wavelengths where chlorophyll has essentially no absorbance. This difference is termed  $\Delta(A_{650}-A_{562})$  and  $\Delta(A_{650}-A_{570})$ , respectively, and is used as a measure of the chlorophyll content of the plant in a given well. It would be possible to  
20        empirically determine the extinction coefficient for  $\Delta(A_{650}-A_{562})$  and  $\Delta(A_{650}-A_{570})$  for chlorophyll in DMSO in order to convert these values to absolute quantities of chlorophyll. However, the nature of the microscreen assay requires only relative (i.e., not absolute) measures of plant growth.

Figure 2 shows a comparison of the biomass and chlorophyll content of each plant.  
25        The excellent fit of the results to a straight line (correlation coefficient,  $r^2=0.91$ ) indicates that chlorophyll content is directly proportional to biomass. Inspection of the data indicates that all but one of the plants closely fits this proportionality. Visual scoring indicates that the single exception was probably due to an error in measuring the biomass rather than the chlorophyll.

30        In order to determine whether the chlorophyll assay would provide a good measure of herbicidal damage, the responses to two herbicides which produce different types of injury were measured both by biomass and by the chlorophyll assay. Damage caused by the herbicide chlorsulfuron is manifested in the microscreen by reduced plant growth. In contrast, the herbicide norfluorazon produces plants which germinate and  
35        have expanded cotyledons, but which lack chlorophyll. Figures 3A and B show the responses of *Arabidopsis* plants grown in the microscreen and treated with a range of concentrations of chlorsulfuron and norfluorazon. In each case, the dose response curve

as measured by the biomass assay is very similar to that measured by the chlorophyll assay.

Other Assays: Other assays for herbicidal damage can also be used, including computerized analysis of images of the plants. This assay should allow the rapid, automated, and non-destructive determination of the size, level of pigmentation and morphology of the plants without removing them from the microtiter plates.

A second assay which can be used relies on the kinetics of chlorophyll fluorescence as a measure of plant health. Like the image analysis assay, this assay will also be rapid, automated, and non-destructive. Unlike most of the other assays, this assay will provide a measure of the health of the plant at the time the assay is conducted, rather than a cumulative measure of the plant health during the course of the growth period. For this reason, it may provide a useful alternative means of quantifying rapid herbicidal damage.

A third assay utilizes reporter genes (e.g. luciferase, beta-glucuronidase, green fluorescent protein, anthocyanin biosynthetic enzymes, etc.) under the control of constitutive, tissue-specific or environmentally inducible regulatory elements. For this type of assay, plants used for the microscreen would be transformed with constructs containing these reporter genes and the appropriate regulatory elements. For example, luciferase activity can be rapidly and sensitively quantified from intact plants grown in microtiter plates in a non-destructive manner using a luminometer. If the luciferase gene is controlled by a constitutive regulatory element, then the total luciferase activity will be dependent on the total biomass of the plant. If a root-specific regulatory element were used to control the luciferase, then the luciferase activity would measure the amount of root biomass present on the plant. If a stress-induced regulatory element is used to control the luciferase, then the stress produced by a particular compound could be measured directly. Stress-induced regulation of luciferase offers the possibility of detecting chemically-induced stress at test compound concentrations which are below that required to produce visible damage to the plant, thus decreasing the amount of compound required for screening.

A fourth assay utilizes expression of the calcium-sensitive luminescent protein apo-aequorin to monitor the level of calcium present in specific compartments and tissues of plants. Variation of calcium levels caused by application of test compounds can be monitored by detection and measurement of luminescence mediated by the reconstituted aequorin. This variation is indicative of changes in cellular metabolism and would therefore serve as a sensitive measure of stress induced by application of herbicidally active compounds.

#### Species Sensitivity to Herbicide Lead Compounds and Greenhouse-Inactive Compounds

The sensitivity of plant species in the microtiter plate assay was evaluated by testing 11 previously selected plant species against a library of 105 compounds known to

have herbicidal activity as demonstrated in traditional greenhouse primary screens. Compounds which demonstrated no herbicidal activity in the greenhouse were also included in the evaluation.

Plates were prepared as previously described. A single seed of a plant species was seeded into a microwell containing 100 uL of 1/2x MS media in agarose containing 10 ppm of test compound or DMSO for a control. Each treatment was replicated 8 times per species (one column) in each test plate.

A total of 105 herbicidally active compounds and 50 compounds which were inactive in traditional greenhouse primary screens were tested in the whole plant microtiter plate assay. Results for greenhouse active chemistry are presented in Table 5, and results for greenhouse inactive compounds are presented in Table 6.

Table 5. Sensitivity of Plant Species to Lead Herbicidal Compounds.

Plant Species	Very Active (3-4 rating)	Active (1-2 rating)	Inactive (0 rating)	% Active
<i>Arabidopsis thaliana</i>	61	37	7	93
<i>Asrostis stolonifera</i>	40	15	50	52
<i>Browallia speciosa</i>	43	20	42	60
<i>Coleus blumei</i>	29	21	55	48
<i>Digitalis purpurea</i>	45	26	34	68
<i>Digitaria sanguinalis</i>	33	26	46	56
<i>Inula Ensifolia</i>	30	37	38	64
<i>Nicotiana tobacum</i>	50	25	30	71
<i>Oxalis stricta</i>	36	29	40	62
<i>Panicum coloratum</i> *	-	-	-	-
<i>Petunia hybrida</i>	58	26	21	80

\* *Panicum coloratum* was not evaluated due to poor germination.



Table 6. Sensitivity of Plant Species to Compounds Herbicidally Inactive in the Greenhouse.

Plant Species	Very Active (3-4 rating)	Active (1-2 rating)	Inactive (0 rating)	% Active
<i>Arabidopsis thaliana</i>	4	12	34	32
<i>Asrostis stolonifera</i>	0	1	49	2
<i>Browallia speciosa</i>	2	3	45	10
<i>Coleus blumei</i>	0	4	46	8
<i>Digitalis purpurea</i>	2	6	42	16
<i>Digitaria sanguinalis</i>	0	1	49	2
<i>Inula Ensifolia</i>	0	3	47	6
<i>Nicotiana tobacum</i>	2	6	42	16
<i>Oxalis stricta</i>	0	10	40	20
<i>Panicum coloratum</i> *	-	-	-	-
<i>Petunia hybrida</i>	6	7	37	26

\* *Panicum coloratum* was not evaluated due to poor germination.

- 5        These data indicate that plants used in the herbicide microscreen were very sensitive biological indicators, with 99% of the lead compounds and 32% of the inactive compounds expressing visually detectable injury to at least one plant species. *Arabidopsis* and *Petunia* are the most sensitive species tested with 93 and 80% of the lead molecules showing herbicidal effects, respectively. *Coleus*, *Asrostis*, and *Digitaria* were generally less sensitive with 48, 52, and 56% of the lead compounds showing activity, respectively. Of the lead compounds tested, 80% were "very active" (visual rating score of 3 or 4) against at least one species, as compared with 16% of the greenhouse inactive compounds.

#### Whole Plant High-Throughput Screen Evaluation

- 15        Seven plant species (*Arabidopsis*, *Browallia*, *Coleus*, *Digitaria*, *Nicotiana*, *Oxalis* and *Petunia*) were selected for final evaluation of the whole plant microscreen based on uniformity of growth, germination, and biological sensitivity to herbicidal compounds determined from the previous experiments.

- 20        Chemical compounds (both active and inactive) were randomly selected from both internal and external sources which had been previously tested in the traditional

greenhouse primary herbicide screen. Plates were prepared as shown above. A total of 14 treatment plates were prepared from each daughter plate for evaluation of seven plant species and two replications of each species.

Data from the 2024 compounds tested in the microscreen are summarized and compared with general greenhouse activity in Table 7.

Table 7. Comparison between microscreen and greenhouse herbicidal activity.

Plate	Micro Scr Advanced <sup>1</sup>	Greenhsc Active <sup>2</sup>	Greenhsc Advanced <sup>3</sup>	Non Micro Grnhsc hits <sup>4</sup>	Non Micro Grnhsc Advanced <sup>5</sup>
1	10	5	4	1(2)	0
2	12	1	0	0	0
3	5	2	1	0	0
4	5	6	2	3(2,2,2)	0
5	9	2	0	0	0
6	7	4	2	0	0
7	17	1	0	0	0
8	8	1	1	1(4)	1 (broadly active)
9	4	2	1	1(3)	0
10	9	5	2	2(1,2)	0
11	8	5	2	1(2)	0
12	10	1	0	1(1)	0
13	10	1	1	0	0
14	10	1	0	0	0
15	14	4	0	2(2,2)	0
16	13	1	0	1(1)	0
17	10	3	0	0	0
18	12	6	3	1(2)	0
19	11	6	0	1(2)	0
20	9	0	0	0	0
21	8	5	0	3(1,1,2)	0
22	13	1	0	0	0
23	11	0	0	0	0
Totals	225	63	19	18	1
% Advanced	11.1	3.1	1.0		

<sup>1</sup>Compounds showing significant activity, i.e., replicated activity on a single species of greater than 2, or moderate activity, i.e., replicated activity on more than one species of less than or equal to 2.

<sup>2</sup>Injury level of 50% or greater on one or more species in greenhouse testing.

<sup>3</sup>Compounds advanced for further evaluation from greenhouse testing which have acceptable activity levels in novel chemical areas or known chemical areas of interest.

<sup>4</sup>The number of greenhouse active compounds (column 3) not advanced in the microscreen (column 2). For those compounds not advanced in the microscreen, the number in parentheses indicates a summary of their greenhouse activity using the 0-4 microscreen rating scale.

<sup>5</sup>The number of greenhouse advanced compounds (column 4) not advanced in the microscreen (column 2). For those compounds not advanced in the microscreen, a description of greenhouse activity is provided in parentheses.

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#### Herbicide Waste Stream Reductions

Implementation of the herbicide microscreen has many benefits, including reduced resource and compound requirements, higher throughput capabilities, and a tremendous reduction in the waste stream associated with primary herbicide screening. The material waste stream from the primary herbicide screen can effectively be reduced by >98% using the microtiter plate screening system. Additional environmental benefits include significant reductions in acetone emissions from spray applications and less landfill waste since microtiter plates would likely be incinerated.

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## CLAIMS

1. A method for evaluating at least one compound for its biological activity on plants, the method comprising
  - (a) placing a plant growth medium, at least one compound to be evaluated,  
5 and at least one seed into contact with each other and into a container having a cross-sectional area measured in the horizontal direction of not greater than 1 cm<sup>2</sup>, the at least one seed being a seed which will germinate into a plant of the genus selected from the group consisting of *Ageratum*, *Arabidopsis*,  
10 *Arabis*, *Artemisia*, *Asrostis*, *Browallia*, *Capsella*, *Coleus*, *Cortaderia*, *Cynodon*, *Cyperus*, *Digitalis*, *Digitaria*, *Eragrostis*, *Inula*, *Ipomopsis*, *Laevis*, *Lemna*, *Nicotiana*, *Oxalis*, *Panicum*, *Petunia*, *Platycodon*, *Recta*, *Sagina*, *Santolina*, *Thymophylla*, and *Thymus*,  
15 (b) maintaining the container with the medium, at least one compound and at least one seed under conditions such that in the absence of the at least one compound the at least one seed would germinate and the resulting plant would grow, and  
(c) evaluating the resulting germination and plant growth.
  2. The method of Claim 1 wherein the at least one seed is a seed which will germinate into a plant of the genus selected from the group consisting of *Arabidopsis*,  
20 *Asrostis*, *Browallia*, *Coleus*, *Digitalis*, *Digitaria*, *Eragrostis*, *Inula*, *Nicotiana*, *Oxalis*, *Panicum* and *Petunia*.
  3. The method of Claim 2 wherein the at least one seed is a seed which will germinate into a plant selected from the group consisting of *Arabidopsis thaliana*, *Browallia speciosa*, *Coleus blumei*, *Digitaria sanguinalis*, *Nicotiana tobacum*, and  
25 *Petunia hybrida*.
  4. The method of Claim 1 wherein the cross-sectional area of the container is not greater than 0.5 cm<sup>2</sup>.
  5. The method of Claim 4 wherein the container is one of a plurality of containers substantially the same dimension as each other arranged in a rectangular  
30 matrix on a single supporting plate, and wherein the method comprises placing into each of the plurality of containers a growth medium, at least one seed and either at least one compound to be evaluated or a control material.
  6. The method of Claim 5 wherein the plurality of containers is 96 in number and they are arranged in an 8 by 12 matrix on a standard microtitre plate.
  - 35 7. The method of Claim 1 wherein the container having the medium, the at least one compound and the at least one seed are maintained in an atmospheric environment having an elevated carbon dioxide level of at least about 2200 ppm.

8. The method of Claim 1 wherein the container having the medium, the at least one compound and the at least one seed are maintained in an atmospheric environment having an elevated carbon dioxide level of at least about 5000 ppm.

9. The method of Claim 1 wherein the growth medium and the at least one compound to be evaluated are placed in the container and mixed with each other prior to the at least one seed being placed into the container and into contact with the mixture of the medium and the compound.

10. The method of Claim 1 wherein the results of the evaluation were obtained by visual assay taking into consideration germination of the seed, and size, morphology and color of the plant resulting from the germinated seed.

11. The method of Claim 1 wherein the results of the evaluation were obtained by using an assay which measures chlorophyll content.

12. The method of Claim 1 wherein the results of the evaluation were obtained by using an assay which measures biomass.

13. A method of automatically depositing a seed into each of a plurality of containers, each container having a cross-sectional area of not greater than 0.5 cm<sup>2</sup> and each seed being one that will germinate into a plant of the genus selected from the group consisting of *Ageratum*, *Arabidopsis*, *Arabis*, *Artemisia*, *Asrostis*, *Browallia*, *Capsella*, *Coleus*, *Cortaderia*, *Cynodon*, *Cyperus*, *Digitalis*, *Digitaria*, *Eragrostis*, *Inula*, *Ipomopsis*, *Laevis*, *Lemna*, *Nicotiana*, *Oxalis*, *Panicum*, *Petunia*, *Platycodon*, *Recta*, *Sagina*, *Santolina*, *Thymophylla*, and *Thymus*, the method comprising providing the seeds in at least one fluidized bed, providing a plurality of vertical hollow seed transport tubes each of such tubes having at its lower end an opening the largest dimension of which is smaller than the smallest dimensions of the seed, each of said tubes having means for controlling the air pressure within the hollow portion of the tube, the air pressure within each tube being individually controllable, positioning the plurality of tubes relative to the at least one fluidized seed beds such that the lower ends of the plurality of tubes is partially immersed in the at least one fluidized seed bed, controlling the air pressure within each tube to create a vacuum within each tube sufficient to capture and hold a seed at the opening at the lower end of each tube, positioning the plurality of tubes relative to the containers such that the lower end of each tube is within a container, positioned approximately in the center of the horizontal cross section of each container, below the level of the top of the container and above the level of any contents in the container, controlling the air pressure within each tube to release the vacuum and create pressure within each tube higher than the pressure outside each tube sufficient to release and expel the seed into the container.

14. The method of Claim 13 further comprising having in each container before the seed is expelled therein a substance which when contacted by the seed will inhibit or

prevent further movement of the seed within the container and inhibit or prevent the seed from being expelled from the container due to air currents resulting from any excess air pressure within the tube.

15        15.     The method of Claim 13 further comprising detecting whether each container has had placed therein one and only one seed, and correcting the seeding of any container found not to contain one and only one seed.

10        16.     An apparatus for automatically depositing a plant seed or insect egg into each of a plurality of containers comprising a plurality of vertical hollow seed transport tubes, each of such tubes having at its lower end an opening the largest dimension of which is smaller than the smallest dimension of the seed, each of said tubes having means  
10        for individually controlling the air pressure within the hollow portion of the tube.

15        17.     The apparatus of Claim 16 further comprising a means for automatically detecting whether one and only one seed has been placed in a predetermined target area in each of the plurality of containers, and for recording which containers have not had one seed so placed.

20        18.     A seed transport for use in the apparatus of Claim 16 comprising a hollow cylindrical tube having an outer diameter of 1-4 mm, an inner bore of 0.5-3 mm, having an opening at the lower end of the tube the largest dimension of which is smaller than the smallest dimension of the seed, and the lower end of the tube having a chamfer to deflect seeds away from a seed which is being held at the opening.

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FIG. 1A

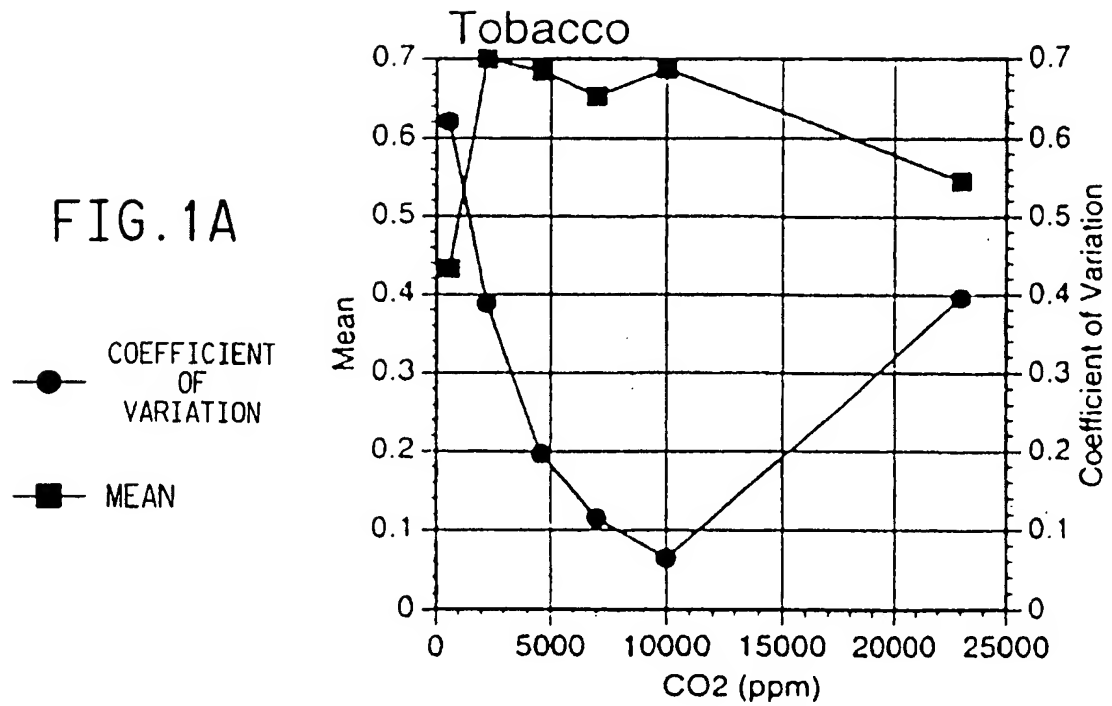
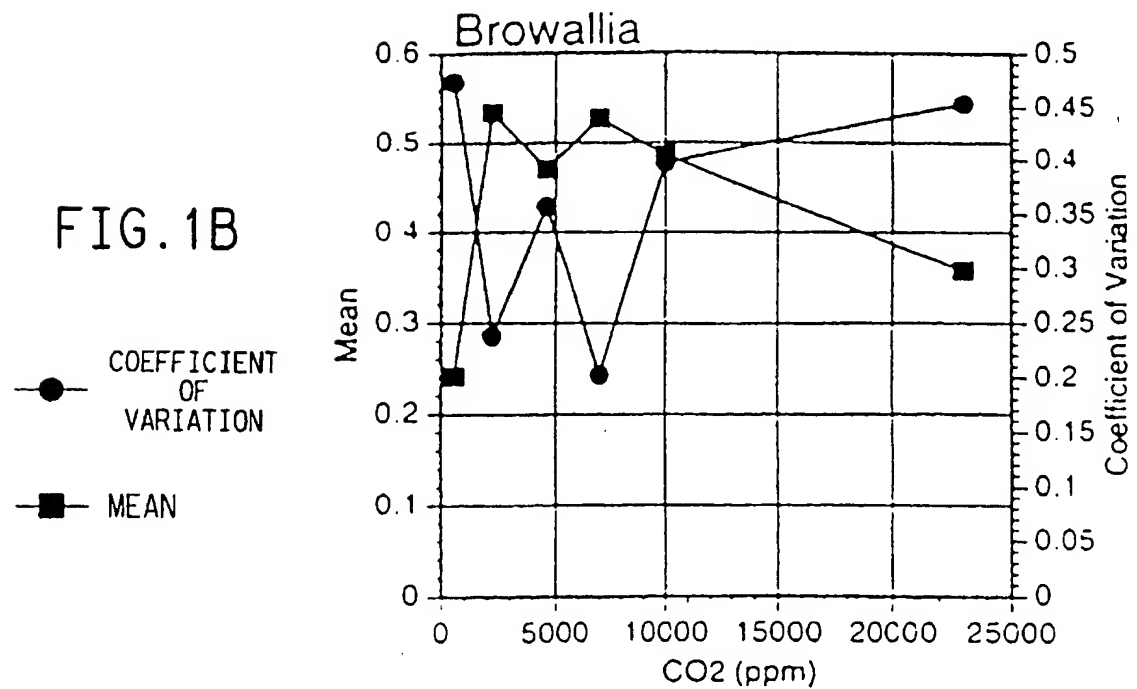


FIG. 1B



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FIG. 1C

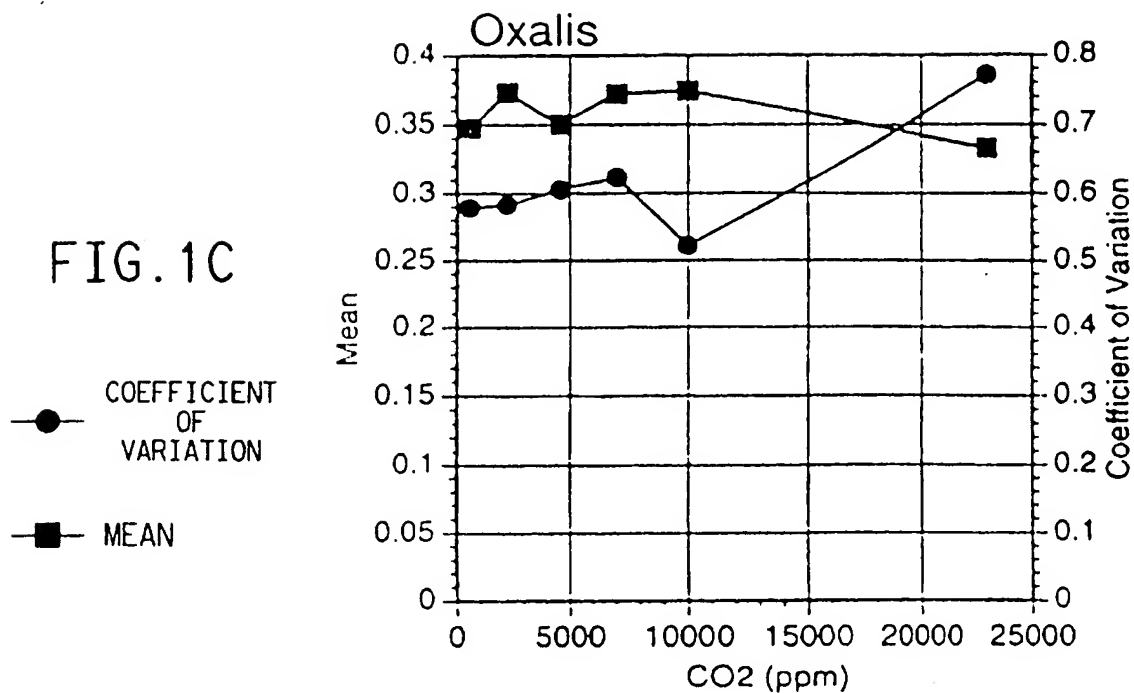
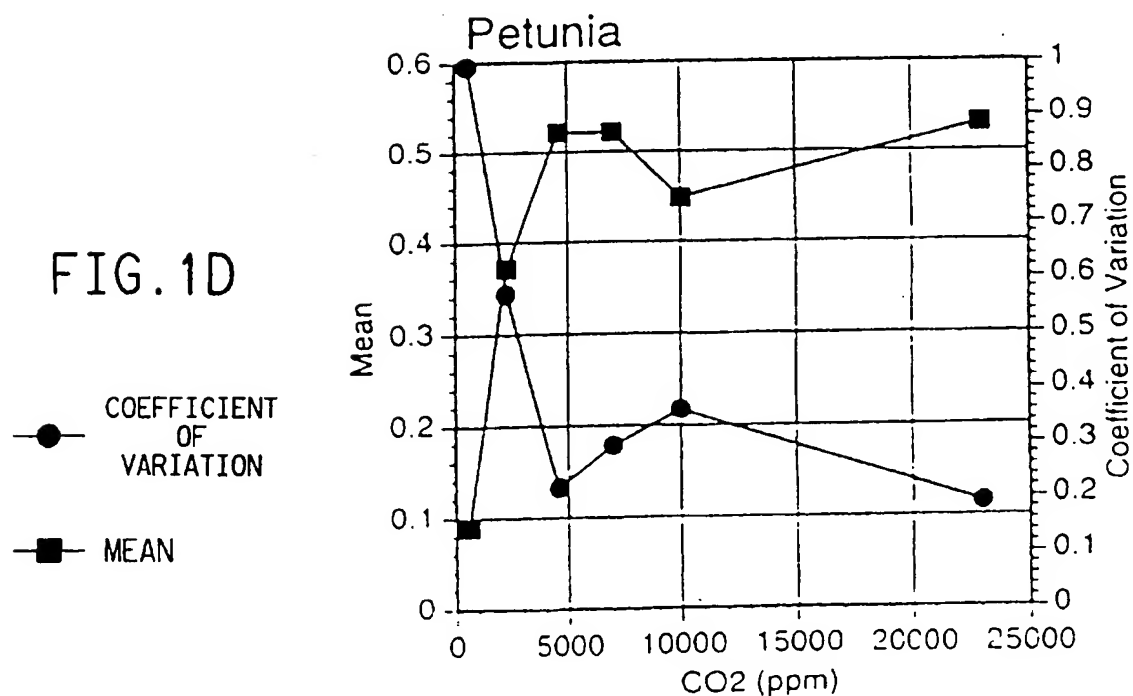


FIG. 1D





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FIG. 1E

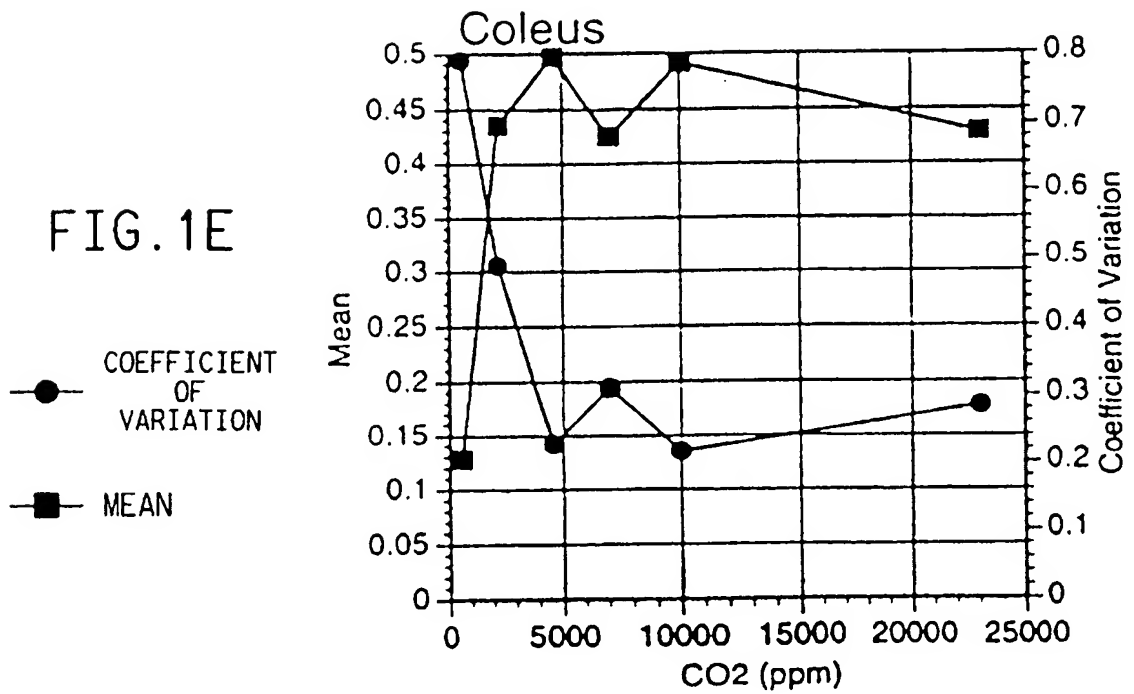
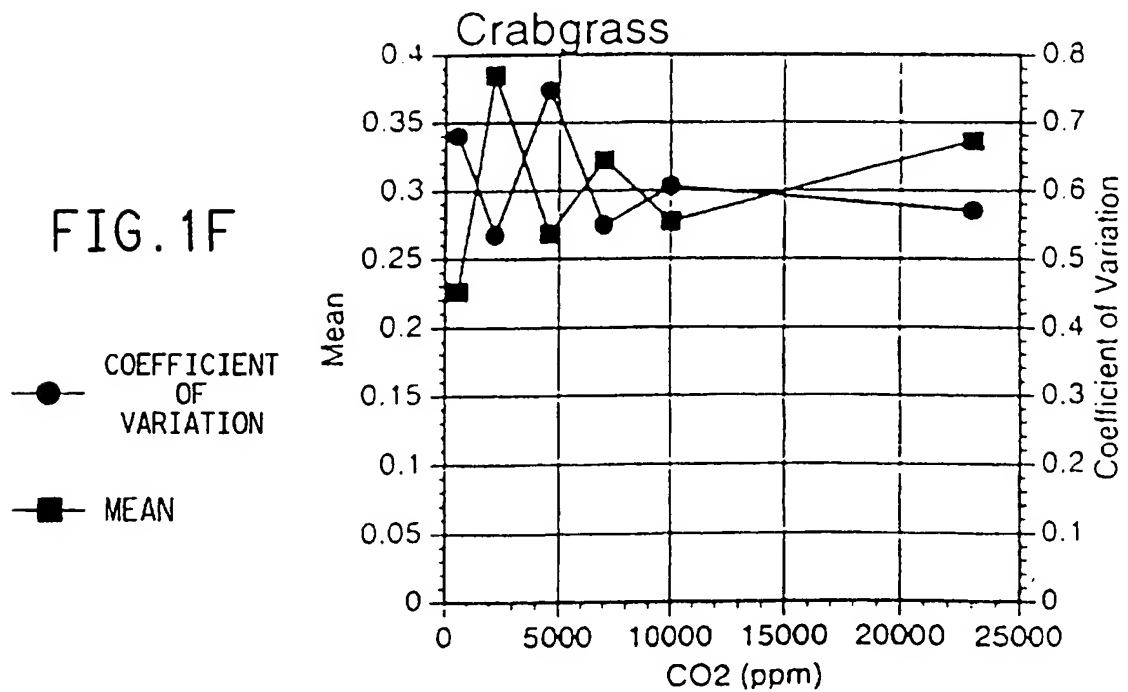
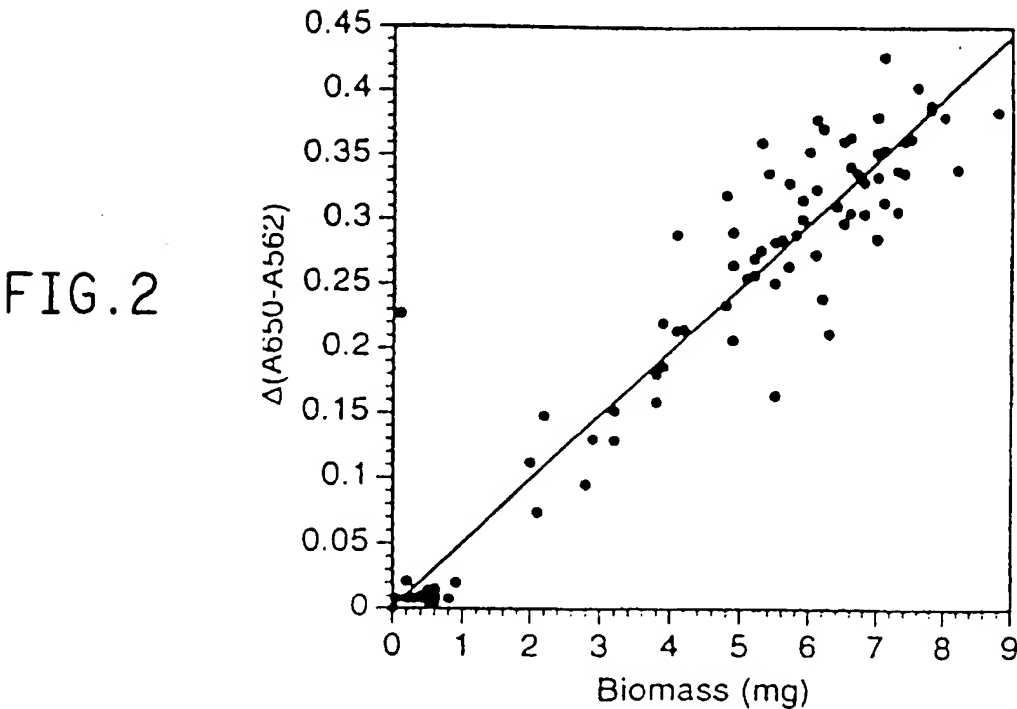
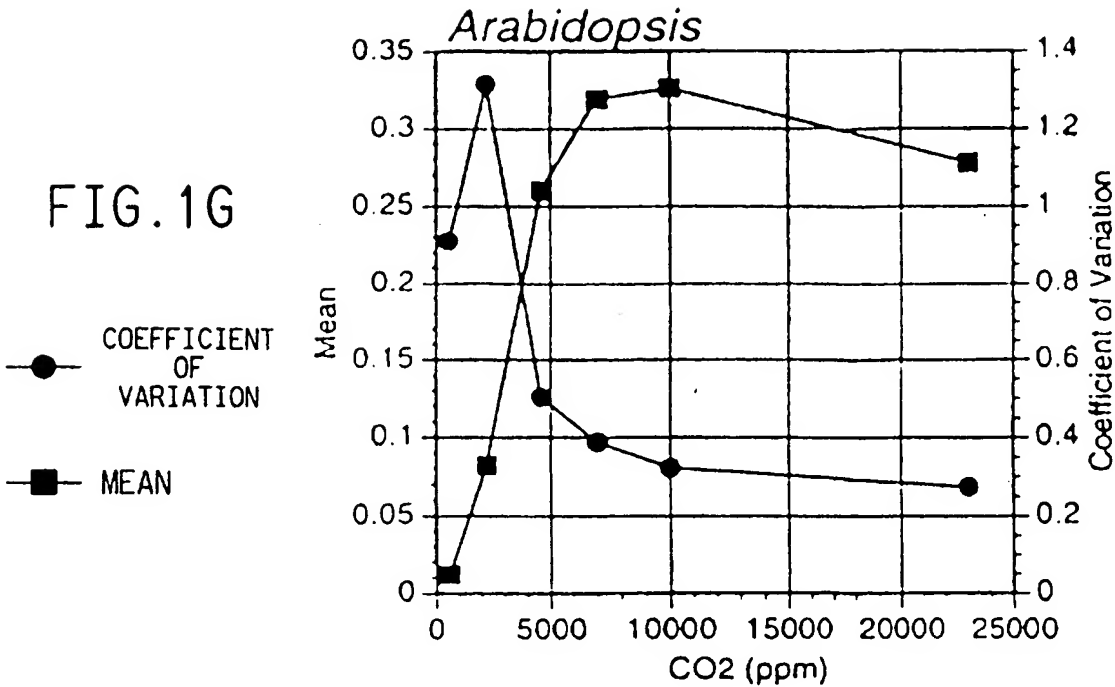


FIG. 1F



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FIG. 3A

■ BIOMASS  
● CHLOROPHYLL

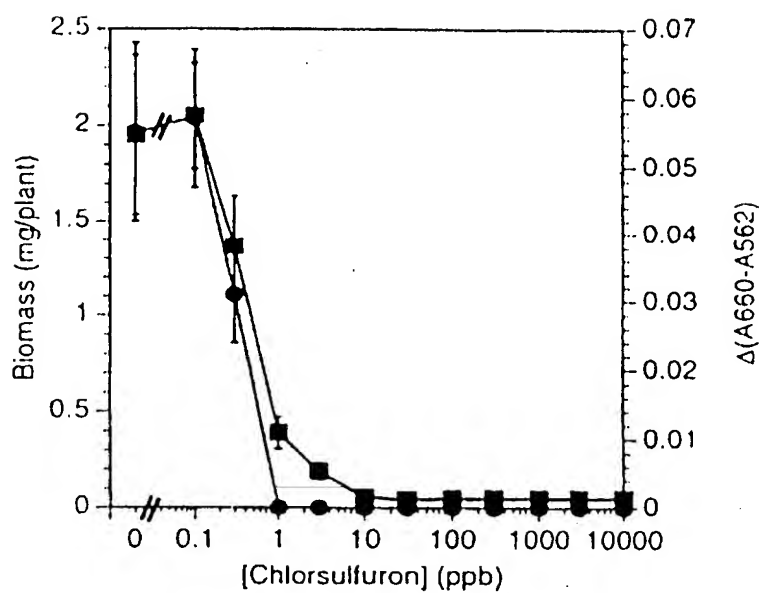


FIG. 3B

■ BIOMASS  
● CHLOROPHYLL

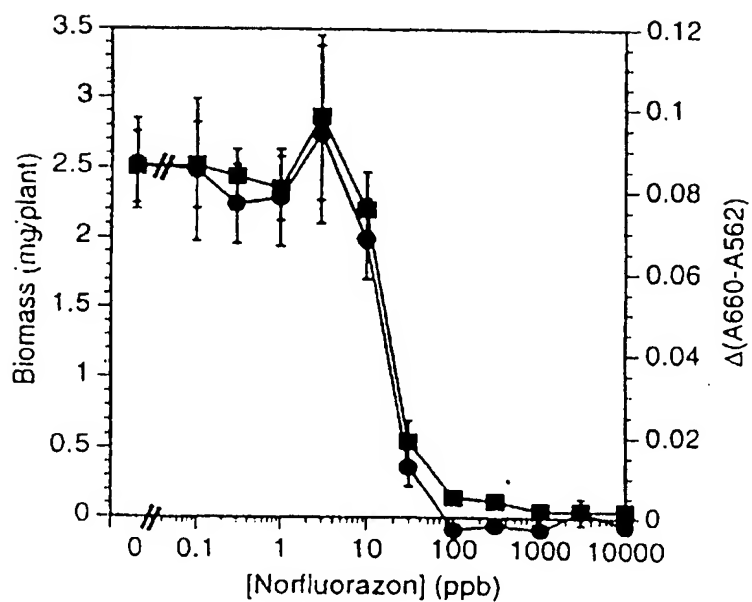


FIG. 4

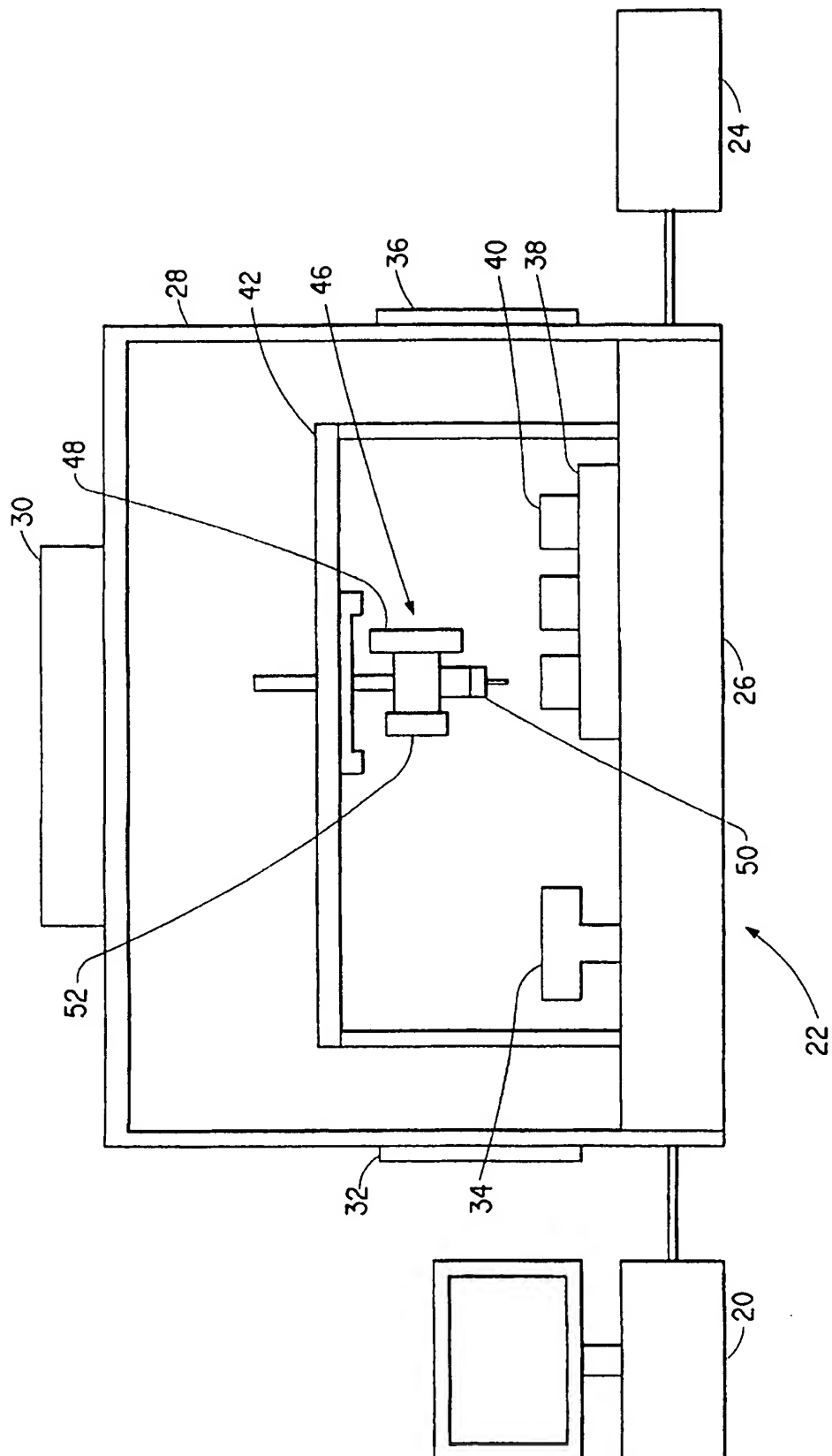
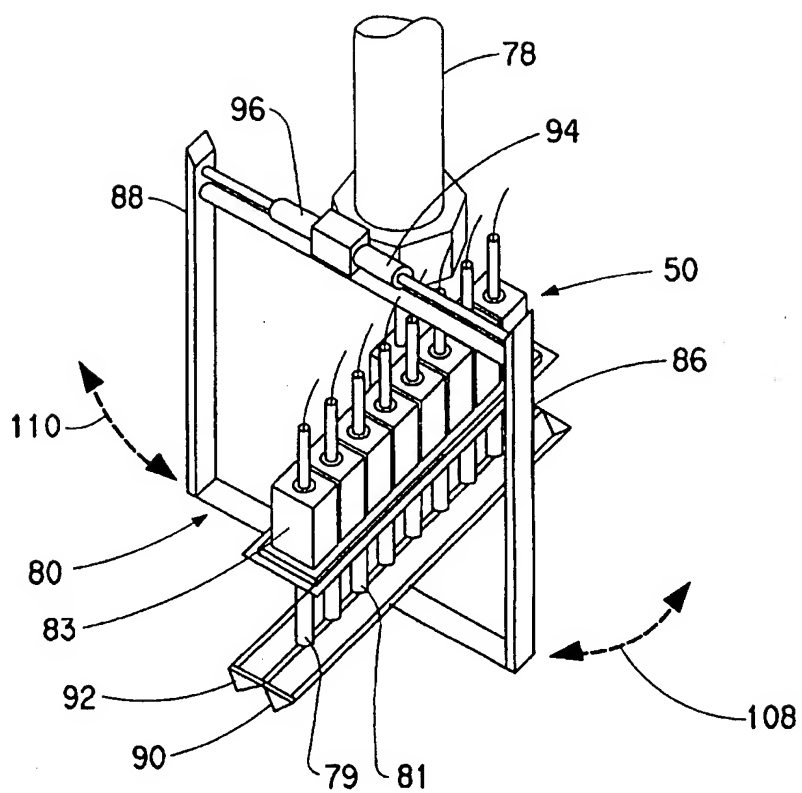




FIG. 5B



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FIG. 6

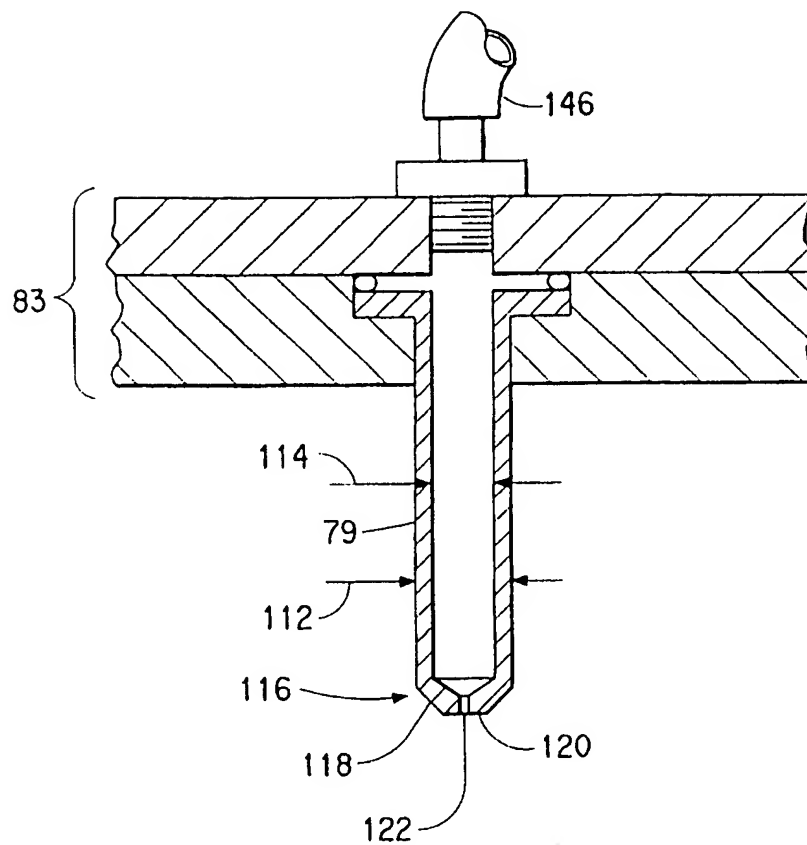


FIG. 7

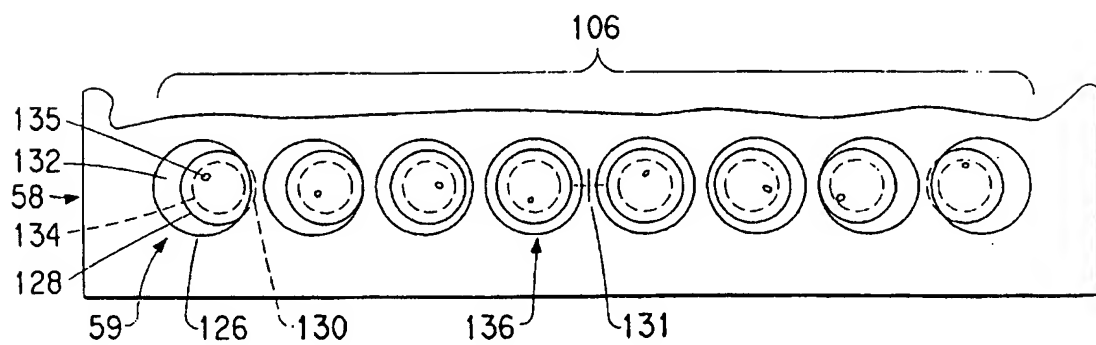
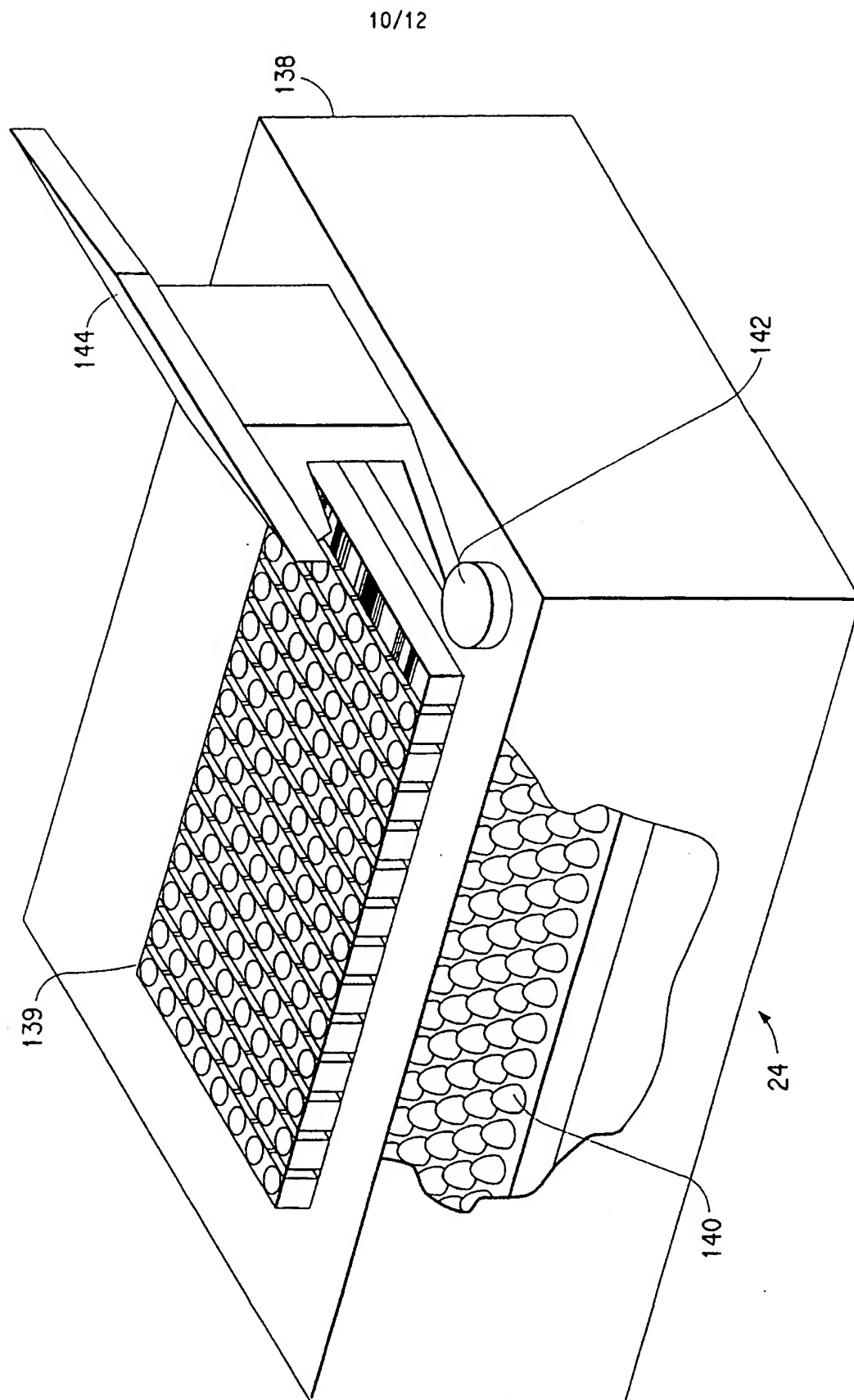


FIG. 8





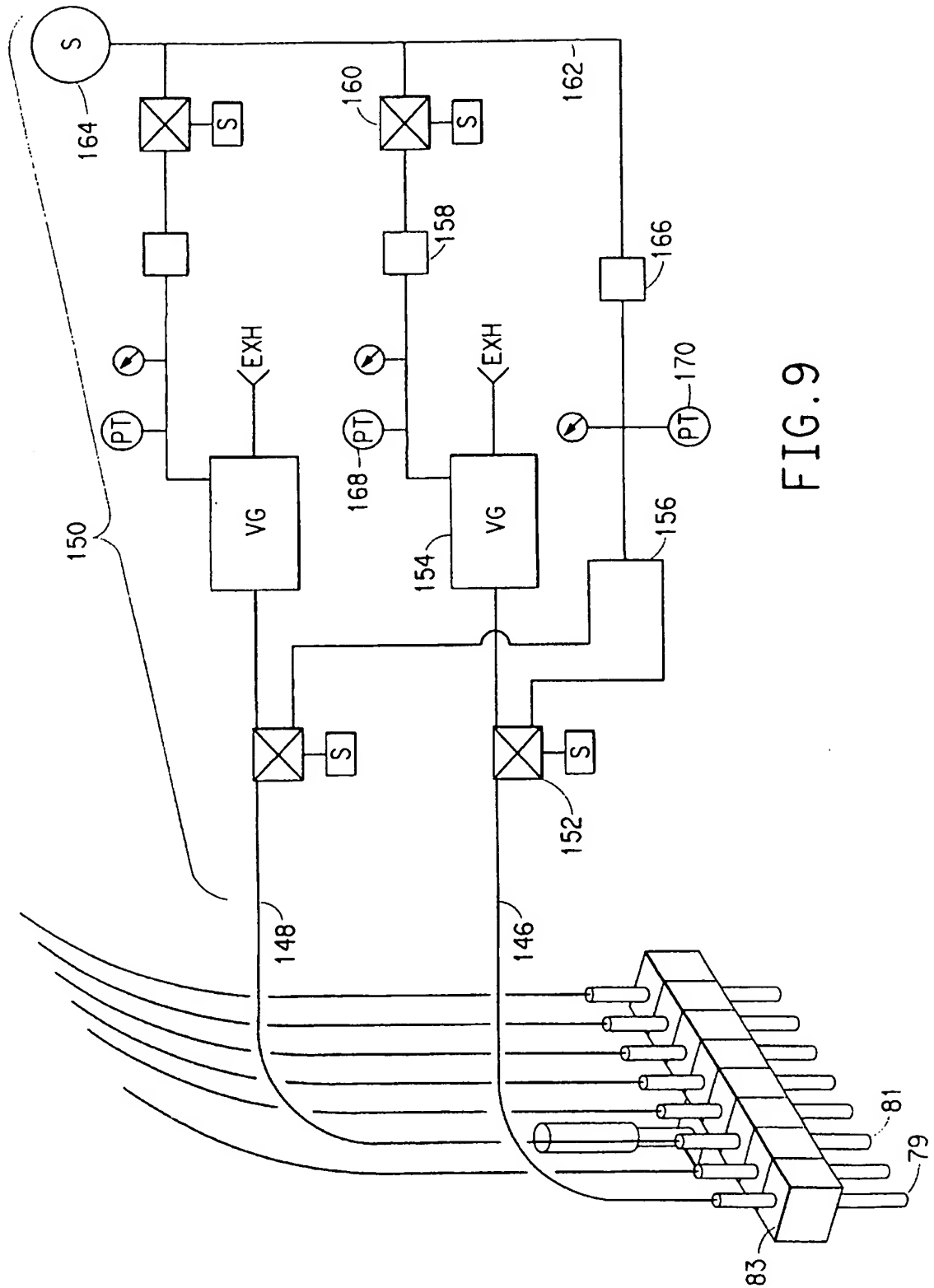
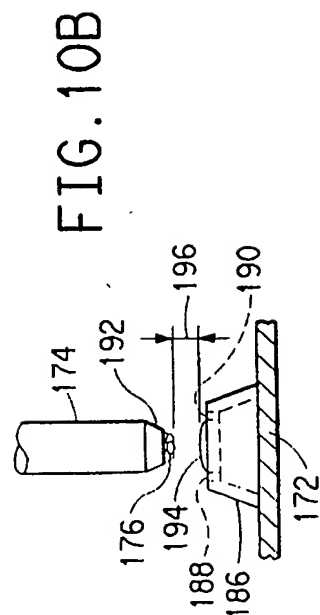
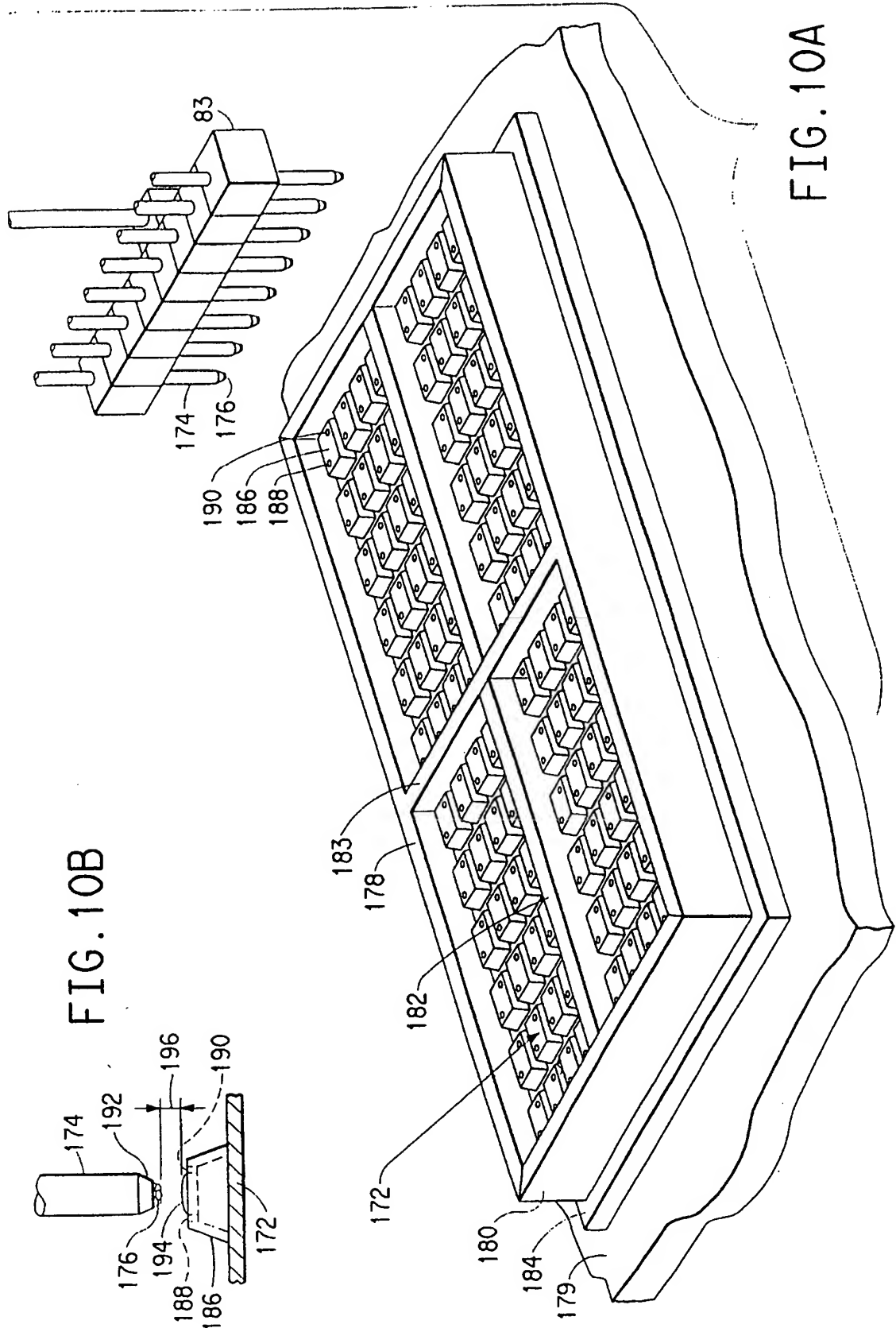


FIG. 9

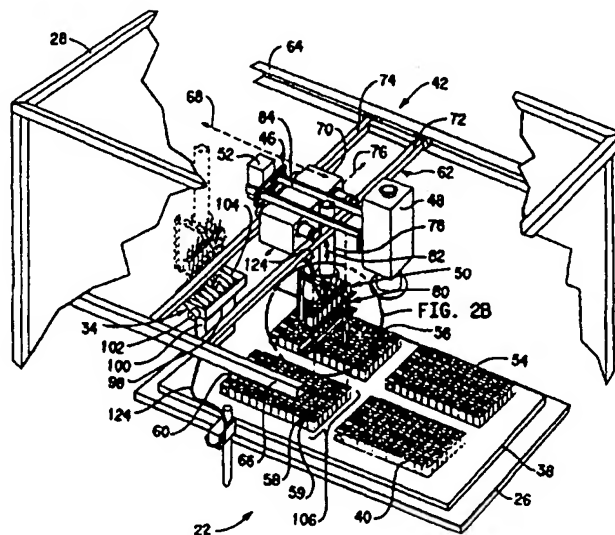




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(22) International Filing Date: 26 November 1996 (26.11.96)			
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(71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): Bhide, Arvind, Krishna [US/US]; 116 Foxfire Drive, Newark, DE 19711 (US). KOEPE, Mary, Kolean [US/US]; 102 Chesterville Road, Landenberg, PA 19350-9600 (US). CASPAR, Timothy [US/US]; 2927 Barley Mill Road, Yorklyn, DE 19736-9702 (US). DIEHL, Kevin, Eugene [US/US]; 56 Vansant Road, Newark, DE 19711-4839 (US). BUSH, Carol, L. [US/US]; 234 Bard Cameron Road, Rising Sun, MD 21911-2631 (US). FRENTZEL, Theodore, William, Jr. [US/US]; 32 Shenandoah Drive, Newark, DE 19711-3767 (US). ROSANIO, Louis, G., Jr. [US/US]; 2712 Landon Drive, Wilmington, DE 19810-2245 (US).		<p><b>Published</b></p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
		(88) Date of publication of the international search report: 28 August 1997 (28.08.97)	

(54) Title: AGRICULTURAL PRODUCT MICROSCREEN METHOD AND APPARATUS



## (57) Abstract

A method and apparatus for screening compounds for agricultural activity has now been developed which employs e.g., intact plants grown in microtiter plates on very small amounts of plant growth media containing a test compound. In comparison to the standard greenhouse screen, the microscreen requires vastly less space, labor, and test compound. However, unlike *in vitro* screens, responses of intact plants are assayed. Using the microscreen, high-throughput screening of test compounds can be accomplished using whole plant responses as the assay.

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/18915

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 G01N33/483 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 897 345 A (HARRIS ET AL.) 30 January 1990 see the whole document	1-3,9,10
A	WO 93 25067 A (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 23 December 1993 see the whole document	1-12
A	DATABASE WPI Week 9502 Derwent Publications Ltd., London, GB; AN 95-008995 [02] XP002027412 & JP 06 292 461 A (JAPAN TOBACCO INC) , 21 October 1994 see abstract	1-12



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Patent family members are listed in annex.

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Date of the actual completion of the international search

12 March 1997

Date of mailing of the international search report

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 8413 Derwent Publications Ltd., London, GB; AN 84-080089 [13] XP002027413 & SU 1 020 067 A (UKR PLANT PROTECT) , 30 May 1983 see abstract ---	1-12
A	DATABASE WPI Week 8701 Derwent Publications Ltd., London, GB; AN 87-005786 [01] XP002027414 & SU 1 230 485 A (CORN RES INST) , 5 January 1986 see abstract ---	1-12
A	FR 2 661 068 A (FERTIL SA) 25 October 1991 see abstract; figures 1,2 -----	5

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 18915

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-12: method of evaluating a compound in its biological activity on plants
2. claims 13-18: method/apparatus for automatically depositing seeds in containers

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
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1-12

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- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/18915

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4897345 A	30-01-90	NONE	
WO 9325067 A	23-12-93	AU 4538293 A	04-01-94
FR 2661068 A	25-10-91	NONE	